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Using *C. elegans* to investigate the transgenerational effects of ethanol and to genetically repair a gait transition impairment in dopamine-deficient animals

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Using *C. elegans* to investigate the transgenerational effects of ethanol and to genetically repair a gait transition impairment in dopamine-deficient animals

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Abstract

Using *C. elegans* to investigate the transgenerational effects of ethanol and to genetically repair a gait transition impairment in dopamine-deficient animals

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The nematode *Caenorhabditis elegans* offers several valuable tools for studying a range of topics in neuroscience and genetics. In this thesis, I specifically exploited the genetic tractability, short generation time, and well-characterized locomotor behaviors of this roundworm to carry out two different projects.

For my first project, I investigated the transgenerational effects of ethanol exposure in *C. elegans*. Parental exposure to certain environmental triggers (stress, toxins, etc.) can alter the phenotypes of unexposed offspring, sometimes persisting for multiple generations. Because alcohol use disorders in humans have a heritable component which is not yet fully understood, I set out to test the effects of ethanol (EtOH) exposure on EtOH sensitivity in subsequent generations. We tested nine cohorts that included an EtOH line derived from female hermaphroditic worms continuously exposed to 24 hours of EtOH during beginning adult stage, as well as a Control line derived from untreated worms. We found that first, second and third generation worms (F1-F3) in the EtOH line showed a minor trend toward

resistance to intoxication relative to the Control line. We also tested four cohorts exposed to EtOH for the same time window but intermittently, and found that worms in the EtOH line showed a trend towards hypersensitivity relative to control. I discuss the complexities of these weak transgenerational inheritance patterns, and how they may be influenced by environmental, timing and testing factors to be considered for future investigations.

For my second project, I uncovered novel mutations that suppressed a locomotor defect in dopamine-deficient worms. Without dopamine, these worms exhibit motor deficits analogous to those in humans with Parkinson's Disease. Dopamine-deficient mutant worms are temporarily immobilized when attempting to transition from swimming to crawling. By conducting a forward genetic screen on the dopamine-deficient mutant *cat-2*, I isolated new mutants that suppressed the abnormal motor transition in these animals. Further behavioral and pharmacological characterization of dopamine-dependent phenotypes of these *cat-2* suppressor (*ctsp*) mutants suggests how their physiology may be altered to suppress the motor transition defects. Future mapping and cloning of the causal suppressor mutations may reveal new targets for treating Parkinsonian motor deficits.

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CHAPTER ONE: INTRODUCTION

This dissertation harnesses the versatility and power of the nematode *Caenorhabditis elegans*. This roundworm has been used for decades now to conduct genetic research, but it continues to expand its scientific repertoire to other areas of research. Among many other fields, *C. elegans* has been contributing to the field of neuroscience for some time now, in spite of the lack of a "proper" brain, itself. Here, I describe how I pursued two very different topics in neuroscience and genetic research using this model in order to advance our knowledge of conserved mechanisms between humans and invertebrates like *C. elegans* which may also have applications in human health. Since these two research projects are so distinct from one another, this introduction is divided into two major sections.

The first section of this introduction outlines background information relevant to the project described in Chapter 2. This project investigates how parental exposure to both chronic and intermittent treatment with ethanol influences ethanol sensitivity in subsequent generations, which are naïve to ethanol -treatment themselves. In this section I will review what is known about the sources of the heritability of alcohol use disorders development. From there I will review the approaches that have been used to study alcohol responses in *C. elegans* in the past. This section will also describe the relevant history of epigenetic and transgenerational research and finally outline some of the evidence for transgenerational epigenetic inheritance in *C. elegans*.

In the second section of the introduction, I review previous research that led to the project described in Chapter 3. This project is concerned with repair of specific locomotor defects in dopamine-deficient worms. This defect impairs the initiation of the crawling gait

(used by *C. elegans* to traverse solid surfaces) after swimming. In this section I will review what is currently known about these locomotor gaits in worms and other invertebrates, and how they are biochemically modulated. This section will also touch on the relevance of this study to human motor disorders cause by dopamine deficiency, such as Parkinson's disease.

PART 1

Heritability of alcohol use disorder

Alcohol is the most widely abused substance in the United States (Hasin, et al 2007). It is fairly common knowledge that alcohol use disorders (AUD) often run in families. Easily observable anecdotal evidence has supported this idea for some time, but a series of familial, adoption and twin studies during the 20th century studied inheritance patterns of AUD and other substance abuse susceptibility appear to confirm that approximately 40-60% of the risk of developing this disorder is genetically inherited (Bierut, 2011; Nurnberger, 2004). Although the genetic contribution has an undeniable influence on AUD development, this means that nearly half of the risk must be attributed to other factors.

Development of AUD is not a one-step process. The progression involves most, if not all, of the following stages: initiation and continuation of alcohol intake, tolerance, and dependence. Early stages of AUD are typically more strongly influenced by environment, while the transition to dependence has a stronger genetic component (Vink, Willemsen, & Boomsma, 2005).

Genes that seem to contribute to AUD risk tend to influence several categories *of intermediate phenotypes*: alcohol metabolism, impulsivity (sensation seeking and behavioral inhibition), alcohol response level (sensitivity) to the effects of alcohol, and major psychiatric conditions (Schuckit, 2014). Of these three classes of genes, those responsible for alcohol response level are the most approachable for study using *C. elegans*. Below I describe some of the alcohol-related behaviors that have been studied in the worm.

Alcohol behaviors in C. elegans

In *C. elegans* we are unable to model certain aspects of addiction, as worms will not typically consume EtOH voluntarily. Therefore, we cannot design experiments that measure volitional EtOH intake. However, worms do display two important characteristics of addiction: tolerance and withdrawal (Bettinger, Leung, Bolling, Goldsmith, & Davies, 2012a; Scott et al., 2017a). During initial exposure to EtOH, worms exhibit a dose-dependent decrease in locomotor output. After continuous exposure to constant dose of EtOH over the span of several hours, locomotor output begins to approach baseline levels. It again decreases when these tolerant worms are removed from EtOH, but baseline locomotion can be recovered during this withdrawal state with the reintroduction of EtOH (Scott et al., 2017a).

EtOH-induced intoxication in worms can be measured using several behavioral outputs, which are dose-dependent: egg-laying, locomotion, and posture. Genetic screens for resistance to intoxication (using locomotion as a measure of intoxication) have identified loss-of-function mutations in a single gene, *slo-1* (Z.-W. Wang, Saifee, Nonet,

& Salkoff, 2001). This gene, named after *Drosophila* ortholog *slowpoke*, is required for EtOH intoxication in worms and encodes for the BK channel – a large-conductance, Ca(2+)-activated K(+) channel encoded by the *slo-1* gene in *C. elegans*, (Davies et al., 2003). Recently, orthologs of loci implicated in AUD (through a human genome-wde association study) were investigated in several different animal models in order to assess their role in ethanol response behaviors. Using locomotion as a measure of intoxication once again, researchers found that three of the loci tested either reduced ethanol sensitivity or impaired acquisition of acute functional tolerance in *C. elegans*. Orthologs of these same three loci affected ethanol response behaviors in flies and mice. These previous studies demonstrate that using locomotion to measure ethanol response behaviors in the worm can allow us to successfully uncover genes that contribute to ethanol sensitivity. It can also be used to test candidate genes implicated in AUD. The reliability of this measure of ethanol sensitivity led me to using it to evaluate epigenetic modulation of ethanol response behaviors across generations.

What is epigenetics?

Broken down literally, the term epigenetics means "over," "upon," genetics. Interestingly, at the time the term was first used, DNA was not yet known to be the vehicle for transmission of genetic information.

The terms "epigenetics" and "epigenotype" were originally coined by Conrad Waddington in 1942 to describe the complex developmental events that bridge the gap between genotype and adult phenotypes. Waddington's model likened the path from gene

to phenotype to a marble rolling down a sloping, hilly terrain, or "epigenetic landscape," made up of peaks and valleys. These peaks and valleys create successive bifurcations forming branching paths that lead to various endpoints at the bottom of the slope, where the marble will eventually come to rest. Through a concept Waddington referred to as canalization an organism that has been subject to natural selection should be able to produce normal phenotypes in spite of slight variations in environment or genotype. Canalization can be imagined as the marble repeatedly rolling along the same specific path (leading to a particular phenotype), deepening the "canal" in the epigenetic landscape, making the phenotype resistant to typical perturbations. Waddington theorized that in the event of highly abnormal perturbations of environment or genotype, new, adaptive phenotypes could arise and then become so deeply canalized that the new traits would persist even in the absence of the causative conditions. He referred to this concept as "genetic assimilation." In a later publication (Waddington, 1952) he attempted to demonstrate this concept using *Drosophila melanogaster*. Waddington found that heat shock of pupa produced a number of adult flies displaying the wing phenotype called crossveinless. After multiple generations of post-heat shock selection of crossveinless flies he found that the selected line he had created had begun producing the mutant phenotype in the absence of heat shock. In reality, this experiment likely was the result of artificial selection for some variant already present in the stock gene pool.

Historical assertions of the possibility of inheritance of acquired traits (Darwin's Pangenesis, Lamarckian inheritance) have largely been dismissed by biologists. Unlike these theories of soft inheritance, Waddington attributed this genetic assimilation to the

new canalization of the crossveinless developmental outcome, which continued to deepen with repeated selection. Finally, when the new "canal" had deepened, it too became resistant to perturbations, and thus the crossveinless phenotype persisted, rather than reverting back to the wild-type developmental canal.

By Waddington's original definition, epigenetics describes all developmental events (that are regulated by genetic input) from fertilization to developmental maturity. This is a much broader definition than the current accepted definition, which is still quite broad. While the term epigenetics now encompasses a vast assortment of molecular gene regulatory mechanisms, it is generally defined as a molecular event that causes a relatively stable change in gene expression that produce a phenotype, without any changes to the underlying DNA sequence. Some versions of this definition also specify that this is the result of interaction between genes and environment (Maze & Nestler, 2011). Dynamic environmental conditions throughout the life of an individual give rise to continuous reshaping of the epigenetic landscape. Although not all mechanisms are known or fully understood, epigenetic modifications that have been described at this time are DNA methylation, histone post-translational modifications (HPTM), and small non-coding RNAs. These modifications are highly interactive and regulate gene expression by either altering the accessibility of the DNA (chromatin remodeling, regulation of transcription factor binding) or by interfering with mRNA transcripts (Gapp, von Ziegler, Tweedie-Cullen, & Mansuy, 2014).

DNA methylation is probably the most widely studied of the three epigenetic modifications. DNA methylation of CpG (cytosine-guanine dinucleotide) islands is

essential to a number of important biological processes in mammals. This epigenetic mark generally represses transcription by blocking transcription factors and recruiting co-repressors complexes at gene promotor regions. As mentioned above, epigenetic mechanisms are interactive, and in the case of DNA methylation, it can lead to the recruitment of histone deacetylaces (HDACs), reinforcing the repressive effect through chromatic condensation (Maze & Nestler, 2011). In mammals, DNA methylation is involved in quite a few key biological processes, including, but by no means limited to: genomic imprinting (for parent-of-origin identification), X-chromosome inactivation, regulating tissue-specific gene expression, transposon silencing, and of course dynamic responses to a wide range of environmental triggers (Maze & Nestler, 2011; Suzuki & Bird, 2008; Zucchi, Yao, & Metz, 2012). Environmental perturbations of various types throughout the lives of mammals can lead to changes in DNA methylation, either in the epigenome of the exposed individual, or in their offspring (or both). Just a few representative examples include stress (Weaver et al., 2004), nutritional deprivation (Heijmans et al., 2008), drugs (Novikova et al., 2008) , and toxins (Crews et al., 2007). It should be noted that this modification, as it occurs in mammals, does not occur in all eukaryotes. Neither Drosophila nor *C. elegans* use CpG island methylation for epigenetic gene regulation (Suzuki & Bird, 2008). In fungi, methylated CpGs are found in areas of highly repetitive DNA, which differs from how the mark is used in mammals (Suzuki & Bird, 2008). Interestingly, a recent study has found evidence that methylation takes place on adenines in the *C. elegans* genomes. This adenine methylation (6mA) may also function as a heritable epigenetic mechanism. By deleting each of the newly discovered DNA

demethylase (*nmad-1*) and DNA methyltransferase (*damt-1*) genes that appear to regulate 6mA in the worm, they were able to accelerate (*nmad-1*) or suppress (*damt-1*) a known transgenerational phenotype (progressive loss of fertility in the absence of the H3K4 demethylase *spr-5*).

Histone post-translational modifications (PTMs) are another epigenetic mechanism through which genes and environment interact. Modifications added to residues on the tails of some histones, most commonly H3 and H4, serve to regulate chromatin state. There are quite a few different possible modifications, which include acetylation, phosphorylation, methylation, sumoylation, and recently discovered, serotonylation (Farrelly et al., 2019). Depending on the modification and the residue being modified, these marks my either act to prevent transcription (repressive) or allow/initiate transcription (activating/permissive). For example, in *C. elegans*, as well as *Drosophila* and mammals, di- and tri-methylation on histone H3 at lysine 9 and 27 (H3K9me2/3 and H3K27me3) are repressive marks, found in high levels in heterochromatin. Di-methylated histone 3 at lysine 4 (H3K4me2), on the other hand, is a permissive mark, found in euchromatin. These marks Changes in histone PTMs (and the enzymes that modify them), like mammalian DNA methylation, are seen as a result of various environmental triggers, such as drug exposure (Ghezzi et al., 2013; Pandey, Ugale, Zhang, Tang, & Prakash, 2008; Fair M. Vassoler, White, Schmidt, Sadri-Vakili, & Pierce, 2013). Core histones and many of their modifications are extremely well-conserved across species and in plants as well (Heard & Martienssen, 2014). Interestingly, caste-specific behaviors in the ant *Camponotus floridanus* are regulated by histone

acetylation profiles. By adding or removing histone PTMs in these ants, their caste-specific behavior can be reprogrammed (Simola et al., 2016).

Small, non-coding RNAs, like the previous epigenetic mechanisms described, serve as conduit for environmental and genetic interaction. In addition to regulating gene expression at the transcription level in some cases (Billi, 2014; Yao, Wang, & Chen, 2019), ncRNAs can also regulate gene expression post-transcriptionally. Non-coding RNAs that act as epigenetic regulators include short interfering RNAs, microRNAs, and PIWI-interacting RNAs. I touch on these further in the discussion of evidence for transgenerational epigenetic inheritance in *C. elegans*, below.

Transgenerational epigenetic inheritance

Although the term epigenetics most certainly applies to alterations in epigenetic landscape within the lifetime of an individual, some define epigenetics more narrowly. For example, 'An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence' (Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009).

Epigenetic inheritance across generations is a relatively new area of study that has only emerged over the last two decades or so but one that is growing very rapidly. Because there are many as-yet unexplored directions, further expansion of epigenetic inheritance studies is likely. The general approach of epigenetic inheritance research involves exposing the test subjects to some environmental trigger, and testing for expected phenotypic changes in subsequent generations. Like the environmental influences that can alter within-

subject epigenotype, possible triggers for transgenerational inheritance include, but are not limited to, stress, nutrition, toxins and drugs.

An important distinction to make when talking about epigenetic inheritance is the difference between transgenerational and intergenerational inheritance. Parental exposure to the experimental trigger may result in transfer of some phenotype to offspring, or even grand-offspring, though mechanisms that are not truly transgenerational.

In rodent transgenerational experimental designs, the P0 environmental trigger often takes place during gestation of the F1 generation (Fig 1.1). There are likely several reasons for this. First, the prenatal developmental window is an important critical period for epigenetic programming (Gapp et al., 2014). It is beneficial for researchers looking to maximize the potential of inducing stable transmission of epigenetic changes to exploit this vulnerable state. Also, although epigenetic mark erasure in the germ cells and zygote are considered to be an obstacle to intergenerational transmission and transgenerational transmission, in utero conditions and maternal-fetal endocrine interaction via the placenta provide another vehicle for establishment of epigenetic reprogramming of the F1 fetus (Keverne, 2015). Another motivation for this experimental design is to be able to examine three different forms of intergenerational/transgenerational transmission. The F1 generation phenotypes and gene expression will be the result of in utero indirect exposure (intergenerational transmission), while that of the F2 generation will be the result of primordial germ cell indirect exposure (also intergenerational). Finally, the F3 generation will be the only one to potentially show true transgenerational effects from ancestral exposure only (Skinner, 2009).

More recently, interest in testing of paternal transmission of epigenetic inheritance has grown dramatically. Transgenerational experimental designs in which the father is exposed to the environmental trigger, prior to conception of F1 offspring, introduce several ideas to consider. Unlike maternal gestational exposure, F1 offspring would have been indirectly exposed to the trigger only as a germ cell. Because of this, true transgenerational effects could be seen as early as the F2 generation (Fig 1.1).

Of course, it would be possible to carry out a study in which the maternal environmental trigger takes place prior to conception of F1 offspring. However, something to consider is that all oocytes of female mammals are carried with her from birth and exposed to the same environmental influences throughout her lifetime. Males have regular turnover of germ cells, allowing for some experimental control over the environmental exposures experienced by sperm that gives rise to F1. This scenario is actually reversed in *C. elegans*, however. *C. elegans* generally exist as self-fertilizing hermaphroditic individuals. Under typical conditions, less than 1% of all worms will be a male, with higher rates of males when stressful conditions arise. Hermaphrodites produce both sperm during late adolescence, then switch from spermatogenesis to oogenesis in adulthood (Wormbook). The hermaphrodite's lifetime supply of sperm is carried with it beginning in adulthood, while oocytes continue to be generated and fertilized.

Returning to the topic of paternal transmission of environmentally induced phenotypes, recent literature suggests that some epigenetic marks may be retained in the male epigenome (Bohacek & Mansuy, 2015; Gapp et al., 2014). As mentioned above, the paternal line has the advantage of excluding some of the confounds intrinsic to the maternal

line, such as maternal care, in utero environment, and behavioral/social transfer of phenotypes. When considering all of these potential advantages to using the paternal exposure approach, it is no surprise that researchers are now beginning to consider this route for transgenerational studies. That said, it is important not to ignore the fact that maternal factors will still influence the offspring. This is highlighted findings from a recent study showing that mothers of offspring fathered by "less preferable" mates (food restricted males) compensated (behavioral and physiologically) with increased maternal investment

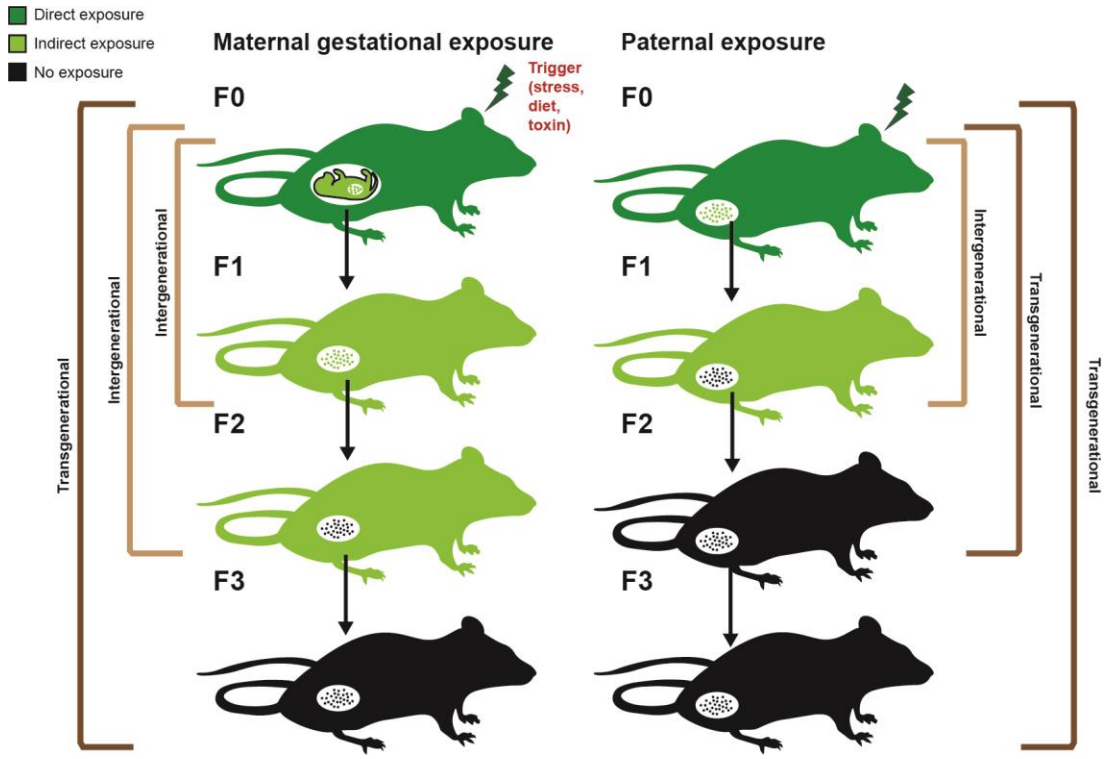


Fig 1.1 - Transgenerational and intergenerational epigenetic effects
Environmental exposure triggers can lead to indirect exposure of subsequent generations either by prenatal exposure or (primordial) germ cell exposure. The diagram shows how each generation is affected by F0 (parental generation) exposure, which differs based on the sex of the parent exposed. Dark green indicates directly exposed animals, light green indicates indirectly exposed animals, and black represents animals not exposed to the trigger prenatally or as germ cells. A heritable phenotype caused the trigger must persist through to the first unexposed generation to be considered transgenerational.

demonstrated by increased gestational weight gain and early increased nursing (Mashoodh, Habrylo, Gudsnuk, Pelle, & Champagne, 2018).

Evidence of transgenerational epigenetic inheritance in C. elegans

Although there is some skepticism in the field regarding strong evidence for true transgenerational epigenetic inheritance in mammals (Heard & Martienssen, 2014), the phenomenon has been demonstrated in *C. elegans* through the use of RNA interference (RNAi).

Gene expression knock-down/silencing by injection of double-stranded RNA (sense and antisense to the target gene) was discovered in *C. elegans* over 20 years ago (Fire et al., 1998) and dubbed RNAi for RNA interference. The exogenous double-stranded RNA is processed to produce siRNA (exo-siRNA) by the some of the same molecular machinery that processes endogenous short interfering RNAs (endo-siRNAs), which are abundant and regulate gene expression throughout the worm genome (Houri-Zeevi & Rechavi, 2017). Both endo- and exo-siRNAs in *C. elegans* can trigger amplification of secondary 22G siRNAs by recruiting RNA-dependent RNA polymerase (RdRPs) to the target mRNA transcripts for silencing. Shortly after the discovery of RNAi (RNA interference) in *C. elegans* researchers discovered that RNAi did not just silence expression of target genes in the animal that had been treated with the dsRNA. Silencing of the target gene could also persist in progeny for multiple generations (Vastenhouw et al., 2006). Although silencing typically lasted 3-5 generations, in rare cases silencing would persist to an extreme degree, upwards of 80 generations. In these cases, however, individuals that

continued to silence the target gene had to be selected in each generation in order to maintain the long-term inheritance. The researchers detected heritable small RNAs in progeny of RNAi-treated parents, as well histone modifications at the targeted loci in the progeny. These small heritable RNAs may have induced these modifications, since they were detected before the chromatin marks appeared (Burkhart et al., 2011). The generational duration of the silencing could also be extended by a administering a second RNAi trigger in progeny of animals that received the first RNAi trigger. Interestingly, the second trigger did not have to be the same as the first trigger (it could target a different gene) to extend duration and potency of the silencing or the original trigger (Houri-Ze'evi et al., 2016). Taken together, much of the heritable RNAi research in worms strongly suggest that stable inheritance of RNAi silencing requires some maintenance mechanism to prevent eventual loss of silencing. This may be the case for endo-siRNA induced TEI as well.

It was later discovered that PIWI-interacting RNAs (piRNAs) have a similar function to endo-siRNAs, but in the germline only. (Ashe et al., 2012). piRNAs are a class of small RNA that are enriched in the germline. In flies and mammals, they maintain transposon silencing in the germline. Like other siRNAs, piRNAs knock down gene expression by targeting mRNA and preventing translation, or recruiting RdRPs to trigger amplification of secondary 22G small RNAs (Rankin, 2015). The silencing cascade by piRNAs can be counteracted by CSR-1, an Argonaut protein. This dynamic balance between piRNA silencing and CSR-1 licensing help regulate gene expression in the *C. elegans* germline (Shirayama et al., 2012).

In addition to cytoplasmic silencing of target genes via the generation of secondary small RNAs (22Gs), siRNA and piRNA pathways also engage in nuclear silencing mechanisms. To do so, an siRNA:Argonaute protein complex translocates to the nucleus, where the complex then associates with the nascent transcript of the target gene and recruits NRDE proteins, which complex with each other and associate with adjacent chromatin. The NRDE protein complex promotes H3K9me3, inhibiting transcription at the target gene locus. The complex also inhibits RNA polymerase II, preventing further transcription of the pre-mRNA. This two-fold silencing mechanism induces nuclear RNAi, which is heritable across generations, and any siRNAs generated in the parental germline will also be inherited.

The characteristics and mechanisms of small, non-coding RNAs described here demonstrate that transgenerational epigenetic inheritance in *C. elegans* is not only possible, but also tunable - although, the specific mechanistic details are still being worked out.

PART 2

In this half of the introduction I will review some of the research topics that are relevant to our study of repairing a gait-switching deficit in *C. elegans*. To do so I will introduce the concept of gait-switching and how it has been studied in several organisms. From there, I will discuss how gait-switching research led to the discovery and further investigation of central pattern generators underlying rhythmic movement. I will then move on to the role of dopamine as a motor output neuromodulator across taxa, and, briefly, how its dysregulation contributes to movement disorders in humans. Finally, I will review what

is known about gait-switching in *C. elegans*, with particular attention to the role of dopaminergic signaling in switching from swimming to crawling in this organism.

Motor gaits and other neural rhythmic behaviors

Virtually all animals that engage in some form of locomotion do so by initiating a rhythmic neural firing pattern which results in a corresponding rhythmic motor output. Such distinct motor patterns are also referred to as gaits. In order to respond to changes in either external or internal stimuli, an animal may alter its motor output either by simply modulating the *rate* of the gait speed accordingly or by switching to another distinct gait entirely. The difference between to these two types of motor responses becomes apparent in the example of a human motion at various speeds on a treadmill. Walking is used at lower speeds and running at higher speeds. These two are distinct forms of motion, and, importantly, they cannot be merged. While one can vary walking speed and running speed, respectively, the two forms of motion do not exist on a single continuum. As a result, at intermediate speeds humans employ alternating bouts of walking and running. Examples similar to this extend to many animals. Horses, for example, display three distinct gaits: walking, trotting, and galloping (Alexander, 2003).

The roundworm *C. elegans* traverses its environment by employing two distinct motor gaits; crawling when navigating across a solid surface or within a sufficiently viscous medium and swimming in a fluid medium. Much like walking and running in humans, the worms alternate between swimming and crawling during intermediate conditions, such as in a medium-viscosity fluid.

It can be challenging to study the neural rhythmic patterns responsible for generating gates in intact, moving animals. Because of this, much of our understanding of the circuitry underlying gaits comes from studying other rhythmic behaviors, such as breathing and digestion, by isolating and manipulating subsets of neurons in convenient animal models. This approach provided valuable insight into the central pattern generators (CPGs) responsible for these rhythmic behaviors, including locomotor output in the form of gaits.

Although gaits and CPGs have been studied in a variety of models, I will limit this introduction to a few animals that were part of some of the pioneering work on these topics. First, I will focus on work carried out using the domestic cat, which provided some of the earliest evidence of CPGs. Additionally, I will briefly describe two invertebrate studies that were essential in identifying the general components of CPG neural circuitry.

In the early 1900s, a series of studies used decerebrate domestic cats to investigate spinal neural control of stepping (Brown 1911, Brown 1914). These decerebrate cats were prepared by separating the cerebellum and parts of the brainstem from the rest of the brain, preventing conscious control of locomotor output in the limbs. When placed on a treadmill, the decerebrate cats were able to engage in spontaneous rhythmic stepping, including walking, trotting and galloping gaits (Forssberg & Grillner, 1973; Forssberg, Grillner, Halbertsma, & Rossignol, 1980). These experiments provided evidence for a CPG in the form of a motor output and sensory feedback neural circuit that is able to act independently of conscious control. This evidence for a CPG in a mammalian system was very

compelling, but identifying the basic and conserved components of CPG neural circuitry in a such a complex nervous system would have been difficult.

By the 1970s, researchers were taking advantage of the simplicity and accessibility of invertebrate nervous system in order to isolate and investigate neural circuits (Kandel, 1970). One such example is in the work on the spiny lobster (*Panulirus*) stomatogastric ganglion. The isolated preparation of the lobster stomatogastric system consists of about 30 neurons. Study of this simple system produced several very valuable insights into the nature of CPGs. One such key finding is that this small network of neurons is able to maintain two different, independent neural rhythms: the gastric cycle CPG and the pyloric cycle CPG (Selverston, Russell, & Miller, 1976). While the rhythmic pattern of the gastric CPG is triggered by upstream neuronal input, that of the pyloric CPG is innate and continuous (Roberston and Moulins 1981, Mamiya and Nadim 2004), yet the two systems utilize many of the same neurons (Katz and Harris-Warrick 1990). Furthermore, these two systems are subject to a number of neuromodulators, including sensory input. The pyloric system firing frequency can also be reduced by dopamine (Kloppenburg, Levini, & Harris-Warrick, 1999). Taken together, this demonstrates that a small, simple system such as this can generate diverse rhythmic patterns.

Going beyond studying CPGs in a completely isolated preparation, researchers have since identified complete behavioral circuits involving CPGs, sensory input, and motor output in other invertebrate systems, such as the leech. Leech neuron size facilitates recording from individual neurons, allowing researchers to correlate individual neuron activity with motor output and identify elements of CPG circuitry underlying gaits.

Semi-intact preparations of the leech have been used to study gaits, which in leech, much like *C. elegans*, includes swimming and crawling. In these preparations, the head and tail of the leech remain intact, while the body region is reduced to only the nerve cord (Kristan & Calabrese, 1976; Nusbaum, Otto Friesen, Kristan, & Pearce, 1987). This preparation allows for stimulation and recording of individual neurons. Even without midbody musculature intact, recording from motor neurons measures "fictive swimming" induced by stimulation of upstream sensory neurons. Such experiments have been used to extensively characterize the leech swim circuit, including its 5 separate systems and their neuromodulators (Brodfuehrer, Debski, O'Gara, & Friesen, 1995; Weeks, 1982). Briefly, the leech swim circuit comprises 5 systems of neuron categories, which include initiators, maintainers, segmental oscillators, and intersegmental coordinators, and motor output. The swim circuit can be initiated by different types of stimuli (pressure, nociception) which are detected by sensory initiator neurons, which synapse onto trigger neurons, setting off a cascade of interactions between the downstream systems. This swim circuit used by the lamprey as well and in both animals the circuit is modulated by sensory input, proprioception, other CPGs, and external application of electrical and chemical stimulation. For example, application of serotonin or octopamine are sufficient to trigger swim initiation

Although extensive study of this gait in these two invertebrates has uncovered the complete circuitry of this CPG, the molecular components and pathways remain largely unidentified. Furthermore, the semi-intact preparation used in the leech is not amenable to study of the relationship between neural activity and actual locomotor output.

The genetic tractability of *C. elegans* makes it an attractive model for investigating the molecular pathways that operate in rhythmic neural circuits such as this one. The worm is also more amenable to studying gaits in freely moving, intact animals. There are also manipulations of the neural circuitry that can be carried out in the worm that are not feasible in other models, such as optogenetic stimulation and laser ablation of individual neurons. Our lab has used techniques such as these in the past to characterize unique motor gaits and transitions in the worm. Because of this, I felt confident in using *C. elegans* to carry out my dissertation work to further our understanding of gait transitions and the underlying molecular mechanisms.

The conserved role of dopamine in motor output and movement disorders

Across a wide range of taxa, dopamine has been shown to modulate motor output in some form. As mentioned above, application of dopamine was shown to decrease firing frequency in the pyloric pathway of the isolated *Panulirus* stomatogastric ganglion (Kloppenburg, 1999). There is further evidence of motor output modulation by dopamine in other invertebrate systems. In both the leech and the sea snail, dopamine suppresses swimming and induces crawling behavior when applied to the isolated ventral cord ganglia of these animals (McClellan, Brown, & Getting, 1994; Puhl & Mesce, 2008). Dopamine is also used to reduce locomotor speed in both zebrafish and land crabs (Martinez, Murray, Leung, & Stefano, 1988; Souza, Romano-Silva, & Tropepe, 2011). Importantly, fellow biogenic amine, serotonin, often modulates invertebrate gaits in a manner that contrasts with dopamine. As mentioned above, application of serotonin initiates fictive swimming

in both the lamprey and leech, and is also required for swim initiation in the lamprey (Brodin, Grillner, & Rovainen, 1985; Hashemzadeh-Gargari & Otto Friesen, 1989; Willard, 1981; Zhang & Grillner, 2000). This evidence, paired with our lab's previous findings in *C. elegans* (discussed later in this chapter), points to a conserved role of these two neurotransmitters in invertebrate gait control, whereby dopamine is involved in crawl initiation and serotonin is involved in swim initiation.

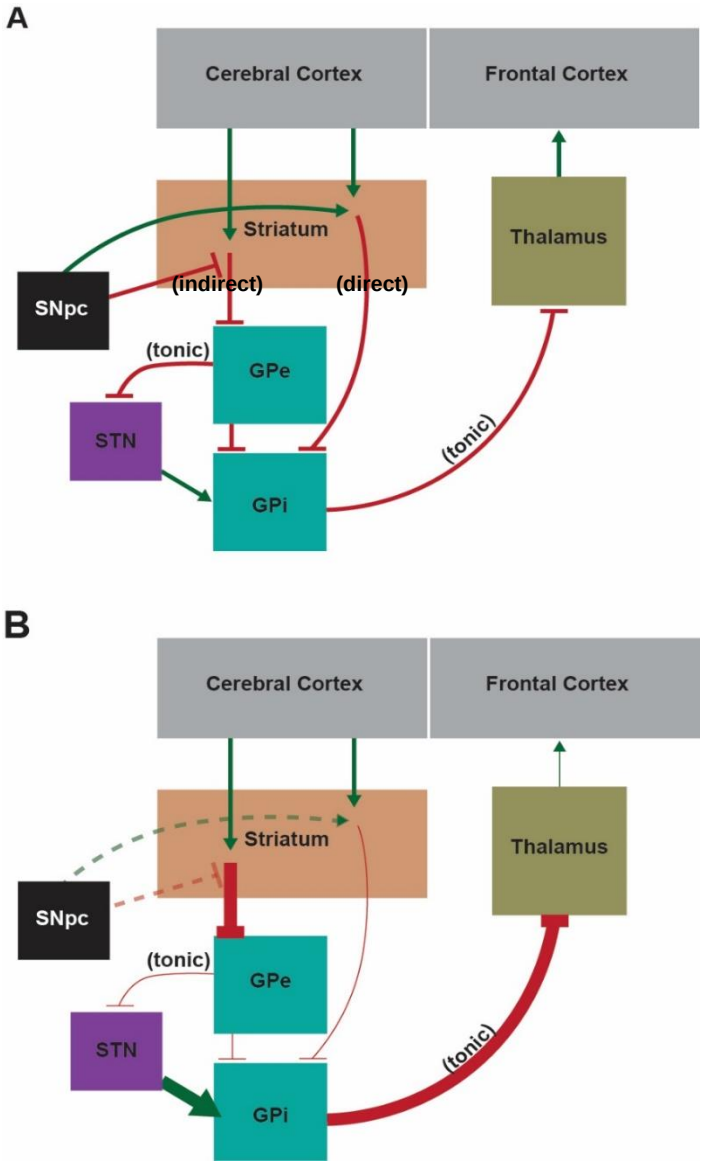
Perhaps the most well-known association between dopamine and motor control is that which is seen in human movement disorders caused by lack of a specific type of dopamine signaling. Parkinsonism (also called Parkinsonian syndromes) is a term that describes a category of human movement disorders that resemble Parkinson's disease (PD). First described by James Parkinson in his clinical writings, "An Essay On The Shaking Palsy," Parkinson's is now recognized as the second most common neurodegenerative disease in humans.

The hallmark of PD neuropathology is progressive loss of dopamine neurons originating in the substantia nigra pars compacta (SNc), which project to the striatum in the basal ganglia (Fig 1.2). There is not a singular cause of dopamine neuron degeneration in Parkinsonian syndromes, and even PD itself falls into the category of "Idiopathic parkinsonism," (Srivanitchapoom, Pitakpatapee, & Suengtaworn, 2018) which means that any given case may stem from one of several known causes, or an unknown cause. Some of the known factors that contribute to PD include: 1) Lewy body formation caused by aggregation of the neural protein α-synuclein, 2) genetic predisposition (often, but not always, caused by mutations or variants in the α-synuclein (*SNCA*) gene or α-synuclein

degradation pathway genes), 3) aging, 4) exposure to toxins, pesticides or heavy metals, and 5) traumatic brain injury (Srivanitchapoom et al., 2018). PD and parkinsonism may cause both motor symptoms - which are primarily caused by the loss of dopamine neurons - and non-motor symptoms. Given the scope of this thesis, background discussed in this chapter will be limited to only topics relevant to motor symptoms.

The primary motor symptoms associated with PD and parkinsonian syndromes include, but are not limited to: tremors, bradykinesia (slow movement), dyskinesia (involuntary, uncontrolled, abnormal movements), shuffling gait, and rigidity. Motor symptoms in later stages, also called axial symptoms, include postural instability, freezing of gait, and difficulty speaking and swallowing. Axial symptoms are typically not responsive to any known treatments. (Coelho & Ferreira, 2012; Connolly & Lang, 2014). Some of the motor features that are impaired in PD are also important in gait transition. For example, tremors, dyskinesia, and freezing gait are the result of inappropriate or inefficient initiation of or termination of motor commands.

Parkinsonian motor deficits resulting from loss of dopamine signaling is not something that is exclusive to humans. Lesion experiments in rodents and non-human primates have confirmed that this a conserved mechanism in some, if not all, mammals (Schober, 2004). Interestingly, the toxin that is most frequently used to induce lesions in non-human primates was actually discovered by accident. In the 1980s, a population of drug addicts began to show acute-onset idiopathic parkinsonian symptoms. Their symptoms were later found to be caused by self-administering a type of "synthetic heroin."



The substance, 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP), is an analogue of the narcotic drug meperidine, also known as Demerol. This neurotoxin is now used in a variety of animal models, including primates, rodents, dogs, cats, sheep and fish (Gerlach and Riederer, 1996).

It should be noted that PD does not appear to occur naturally in any other animal. Through the use of neurotoxins that target dopamine neurons, however, comparable pathological

Fig 1.2 - Schematic representation of the direct/indirect pathway classical model in the physiological condition and in Parkinson's disease
In healthy individuals (*A*), dopaminergic inputs from the substantia nigra pars compacta (SNpc) to the striatum activate the direct and indirect pathways of the cortico-basal ganglia-thalamo-cortical loop through D1 receptor and D2 receptor signaling, respectively. These inputs maintain the proper balance between the direct and indirect pathways needed to disinhibit the thalamus, allowing for appropriate motor output. (*B*) In the brains of individuals with Parkinson's disease, dopaminergic projections from the SNpc degenerate over time. Without these inputs to the striatum, there is no balance between the direct and indirect pathways that are responsible for thalamic disinhibition. Instead, overinhibition of the thalamus impairs normal motor output. Red lines represent inhibitory inputs, green represent excitatory inputs. Differences in line thickness represent changes in the level of signaling between the healthy striatal circuitry and Parkinsonian. STN = subthalamic nucleus. GPi = Globus pallidus interna. GPe = globus pallidus externa.

symptoms can be induced in a range of animals including mice, rats, and non-human primates (Gerlach & Riederer, 1996). These three animals are the most commonly used as a model for PD. Because of the highly conserved neural circuitry of the basal ganglia between humans and these models, lesioning nigrostriatal dopamine neurons is an effective way to mimic parkinsonian motor symptoms, and is typically done using either the neurotoxin 6-hydroxydopamine (6-OHDA) or MPTP (mentioned above).

Levodopa (L-Dopa) is probably the most widely known and widely used PD treatments. Although, initially, L-Dopa is very effective in treating the symptoms of PD, the drug is known to actually exacerbate motor deficits, causing dyskinesia, especially after long term use and increased dosage. Taking a D2-receptor agonist drug can mitigate the effects of the dyskinesia induced by L-Dopa, when they are taken together (Nadjar, Gerfen, & Bezard, 2009). This makes sense, as L-Dopa has been found to upregulate the expression of D1, but not D2, receptor expression. In PD, the dopamine neuron loss leads to imbalance between the direct and indirect pathways of the striatum which are activated D1-expressing and D2-expressing neurons, respectively. If this imbalance is initial treated by L-Dopa administration, through upregulation of D1-like receptors only, the imbalance may shift in the other direction. Adding a D2-agonist helps maintain balance between the two. Below, I describe work in *C. elegans* that similarly supports the notion that dopamine receptor balance is necessary for proper locomotor output.

The role of dopamine in swim-to-crawl initiation in C. elegans

Our lab has previously demonstrated that *C. elegans* displays two distinct forms of locomotion : crawling and swimming (Pierce-Shimomura et al., 2008; A. G. Vidal-Gadea & Pierce-Shimomura, 2012; A. Vidal-Gadea et al., 2011). During crawling, a deep dorsoventral bend is slowly propagated down the length of the body (0.5 Hz), resulting in an S-shaped posture (Fig 1.3). During swimming, shallow bends are quickly propagated back (2 Hz) and timed such that the body forms a C-shaped posture. We have begun to understand the neural basis for these motor transitions. For example, we have found that serotonin and dopamine are necessary and sufficient for switching between swimming and crawling respectively (A. Vidal-Gadea et al., 2011). Specifically, disruption of dopamine neurons through genetic mutation, optogenetic inhibition, and laser microablation prevented normal transition from swimming to crawling. Conversely, activating dopamine signaling through optogenetic activation of dopaminergic neurons was sufficient to cause inappropriate transition to crawling behavior in water. These inappropriate bouts of crawling in water can also be the result of reduced serotonin signaling, as this behavior is displayed by the serotonin-deficient mutant *tph-1* while submerged in water. This demonstrated that maintenance of normal swimming requires the correct balance between, these two neurotransmitters. Serotonin and dopamine deficiencies also have inverse effects on to the onset of transition between the two patterns. For instance, wild-type worms delay swim initiation after 6 second immersion into water, while this is hastened in dopamine-deficient worms (3 sec), and extended in serotonin deficient mutants (20 sec). The converse result was observed for crawl initiation.

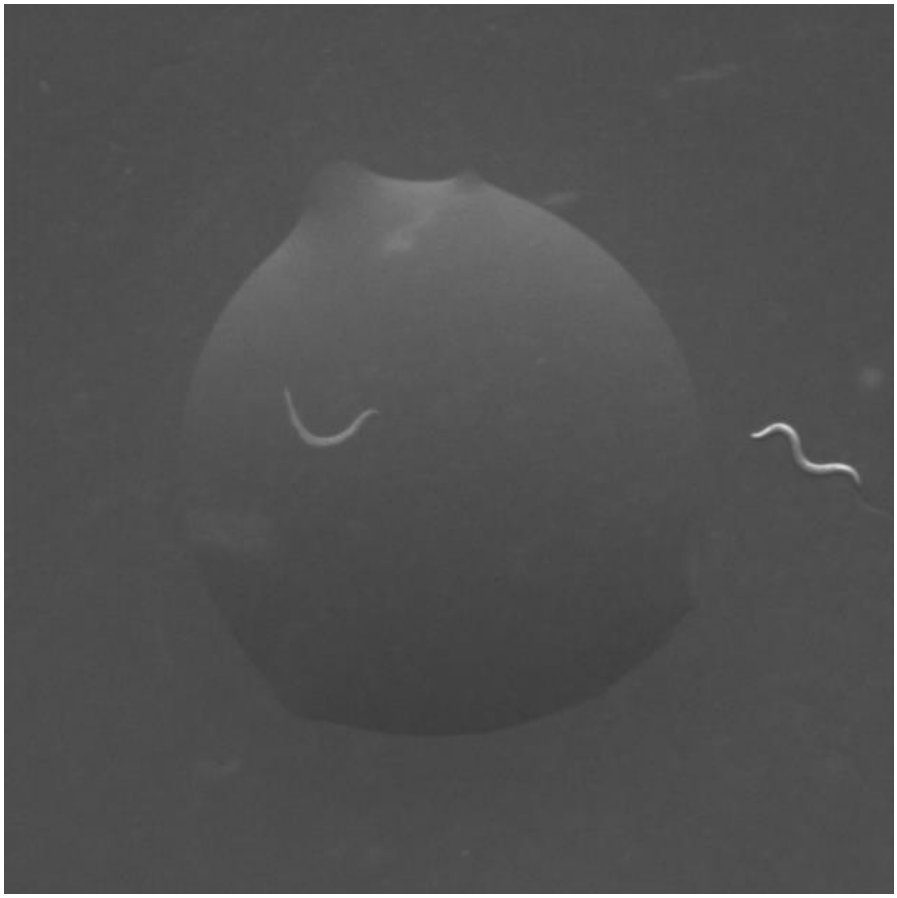


Fig 1.3 - Swimming and crawling are distinct motor gaits in *C. elegans*
The worm on the left is exhibiting swimming behavior, in which the worm takes on a C-shaped posture. The worm on the left is exhibiting crawling behavior, in which the worm takes on an S-shaped posture. Crawling uses a lower frequency of body bends than swimming. The body bends propagate from anterior to posterior and during crawling there may be 2-3 bends being propagated down the length of the worm at once, giving the worm an S-shaped appearance. Swimming uses a higher frequency of body bends, and a single bend affects the entire length of the worm at once, resulting in a C-shaped appearance.

In addition to mutant *C. elegans* deficient in dopamine production, mutant worms lacking D1-like dopamine receptors also show deficits when it comes to transitioning from swim to crawl. Mutants lacking the D2-like receptors did display this impairment, however. In humans, an imbalance between these two receptor types is thought to contribute to Parkinsonian symptoms (Calabresi, Picconi, Tozzi, Ghiglieri, & Di Filippo, 2014). There

is some evidence in mammalian literature that supports this notion as well (Freeze, Kravitz, Hammack, Berke, & Kreitzer, 2013). Thus, our lab has provided the first evidence that such an imbalance may play a role in gait switching in *C. elegans* as well. So far, four dopamine receptors have been identified in *C. elegans*, and are divided into D1-like and D2-like classes, based on homology with human dopamine receptors (Chase & Koelle, 2007). As in humans, D1-like receptors generally produce excitatory responses, whereas D2-like receptors generally produce inhibitory responses, through activation of their associated Gα proteins.

In order to further our understanding of the molecular mechanisms that involved in gait transition, initiation, and maintenance, I continued work based on the previous projects from our lab which investigated neural mechanism underlying swimming and crawling. The goal of this project, which I detail here in Chapter 3, was to uncover gene mutations capable of suppressing the gait-switching defect seen in the *cat-2* mutant. In doing so, I expected to not only to expand our knowledge of how swimming and crawling are modulated on a molecular level, but also to genetically repair a parkinsonian defect in *C. elegans*.

CHAPTER TWO: CHRONIC ETHANOL EXPOSURE IN *C. ELEGANS* MAY CONFER RESISTANCE TO ACUTE INTOXICATION IN NAÏVE PROGENY FOR SEVERAL GENERATIONS

ABSTRACT

Alcohol abuse and dependence has a substantial heritable component. Although the genome had long been considered the sole vehicle of heritable phenotypes, recent studies suggest that drug or alcohol exposure may induce lasting alterations in gene expression that are transmitted across generations. Still, the transgenerational impact of alcohol use (and abuse) remains largely unexplored in part because multigenerational studies using mammals present challenges for time, sample size, and genetic heterogeneity. Here, we take advantage of the extremely short generation time, large broods, and clonal form of reproduction of the nematode *C. elegans* to test whether alcohol exposure alters behavioral responses to acute alcohol treatment in subsequent generations. Using a chronic alcohol treatment paradigm in the P0 generation, we found that naïve F1-F3 progeny of the ethanol-line displayed a trend toward resistance to acute intoxication, albeit insignificant. To test the impact of treatment duration and timing on this transgenerational effect, we repeated the study using a different treatment paradigm: intermittent treatment. Preliminary results from this alternate treatment paradigm shows the inverse of the trend observed following the chronic treatment, suggesting that the duration and frequency of alcohol treatment are key modulators of heritable changes alcohol response in subsequent generations.

INTRODUCTION

Alcoholism is a prevalent and often devastating disease, characterized by a psychological and physical dependence on alcohol consumption. Investigations into familial patterns of alcohol dependence, as well as human twin studies and population genetic studies confirm the considerable heritability of the development of alcohol use disorder (AUD) (Clifford, Hopper, Fulker, Murray, & Rao, 1984; Heath et al., 1997; Kaprio et al., 1987; Partanen, Bruun, & Markkanen, 1966). However, genetic variation alone accounts for only a fraction of the heritability of this condition (Bierut, 2011; Kendler, Prescott, Myers, & Neale, 2003; Nurnberger, 2004). In addition to genetic information, epigenetic modifications that regulate gene expression may also be transmitted to offspring. Epigenetic modifications produce relatively stable changes in gene expression mediated by molecular marks without causing changes to the genetic code itself. Several types of environmental stimuli, such as stress, diet, and toxins appear to be capable of inducing epigenetic modifications, such as chromatin remodeling, DNA methylation, and small RNA activity (Bohacek & Mansuy, 2015; Heard & Martienssen, 2014; Rankin, 2015; F. M. Vassoler & Sadri-Vakili, 2014). In addition to altering the epigenetic landscape of the affected individual, there is increasing evidence that environmental influences can produce heritable epigenetic effects (Casier, Boivin, Carré, & Teysset, 2019).

Rigorous investigation into the phenomenon of transgenerational epigenetic inheritance (TEI) has begun to take off over the last decade. It has been understood for

some time now that epigenetic modifications can be brought on by environmental exposures and that the epigenetic architecture of a cell can be transmitted to its daughter cells (as in mitotic differentiated cells). While it is clear that these epigenetic phenomena occur in somatic cells, the extent of epigenetic plasticity and inheritance in germ cells is still debated. In order for epigenetic information to be inherited by progeny, it must be transmitted through the germ cells. However, in the germ line and early embryos of many animals, including humans, these cells undergo extensive reprogramming, through which DNA is demethylated and chromatin marks are largely, if not completely removed. Similarly, worms undergo a large reprogramming event during early embryogenesis, during which certain H3 histone marks H3K4me2, H3K8 and H3K18 acetylation) are dramatically reduced in germline precursor cells, while others remain stable or increase (H3K36me, H3K27me3)(Kelly, 2014). Therefore, any parental epigenetic "memory" that is to be passed on would have to be resistant to this reprogramming somehow, either by chance through incomplete erasure or with the help of some maintenance mechanism. In spite of this apparent obstacle, publications investigating transgenerational influence of a wide variety of environmental perturbations continue to grow steadily. While some have yielded promising findings, mechanisms for stable transgenerational transmission are still mystery.

Among the relatively few studies of epigenetic inheritance that have focused on drugs of abuse, most use rodent models. (Nizhnikov, Popoola, & Cameron, 2016; Fair M. Vassoler et al., 2013). In this study, to test the transgenerational effects of ethanol on ethanol sensitivity, we turned to the nematode *Caenorhabditis elegans*. This genetically

tractable roundworm possesses several unique qualities that are advantageous for transgenerational work. Perhaps the most useful aspect of using *C. elegans* for any multigenerational study is its very short generation times. The time from laying of the parental (F0) eggs to adulthood of the F3 generation is only 12-15 days. By contrast, using a mouse or rat model, the time from birth of the F0 dam to F3 puberty is approximately 7 months. In addition to carrying out this multigenerational procedure in a fraction of time, each worm produces ~200-300 genetically identical offspring with just a few days. This allows us to quickly propagate hundreds to thousands of individuals each generation, which is essential for collecting sufficient samples for both molecular analysis and behavioral testing. Because the worms self-fertilizing hermaphrodites, no mating is required and working with these clonal populations allows us to confidently attribute phenotypic changes to epigenetic mechanisms. Finally, the worm displays measurable, dose-dependent EtOH response behaviors: reduced locomotion, postural changes and reduced egg-laying.

By exploiting these valuable qualities, we used *C. elegans* to approach the question of how chronic ethanol exposure in the parental generation affects ethanol sensitivity in in subsequent generations. Knowing that AUD risk is heritable and that some of the genes known to contribute to that risk involve alcohol sensitivity and metabolism, we hypothesized that ancestral EtOH-treatment would reduce EtOH-sensitivity later generations. In other words, the EtOH line would show a resistance to acute intoxication. The rationale behind the hypothesis is that an individual that has *increased* sensitivity to alcohol would probably be less likely to develop AUD than someone less sensitive. Furthermore, being able to pass on EtOH resistance to offspring may be particularly

beneficial to worms in the wild, as they feed on bacteria in the soil, which may also coincide with rotting, fermented fruit. Being able to withstand the effects of EtOH would be advantageous in such a scenario.

Using the measure of locomotion as a proxy for alcohol sensitivity, we assessed acute intoxication responses in the ethanol-naïve F1-F3 progeny of chronic EtOH-treated animals as well as untreated controls. In addition to chronic ethanol treatment of the parental generation, we also gathered preliminary data on the transgenerational effects of *intermittent* EtOH-treatment of the P0 generation.

MATERIALS AND METHODS

Worm husbandry

All worms were maintained as previously described (Brenner, 1974). Briefly, worms were raised at 20°C on standard plates which are 6-cm-diameter Petri dishes filled with 12 ml of nematode growth media (NGM) agar seeded with OP50 bacteria. The strain used in this study was N2 (Bristol).

Ethanol treatment

Chronic ethanol treatment. Age-matched P0 (parental generation) worms were harvested during late larval stage 4 (L4) and were divided into a control group and an ethanol treatment group, that will henceforth be referred to as the control-line and ethanol-line, respectively. Chronic EtOH treatment used here was modified from methods previously described in (Scott et al., 2017b). Briefly, treatment plates (for both EtOH treatment and control mock treatment) were prepared by dehydrating seeded NGM agar plates (see worm

husbandry, above) at 37° C with lids removed for 3.5 hours. The EtOH-treatment plate was then prepared from one of the dehydrated plates by placing 280 µl of EtOH beneath the agar to achieve a final EtOH concentration of 400 mM. The ethanol-line was placed on the EtOH-treatment plate and sealed with parafilm. In parallel, the control-line was placed on a mock treatment plate, which was prepared by using one of the dehydrated plates and adding an equivalent volume (280 µl) of water, rather than EtOH, and then sealed with parafilm. Worms remained on their respective treatment plates for 24 hours. This concentration and duration of EtOH-treatment was previously shown to produce worms that have an internal EtOH concentration of ~40-60 mM (Scott et al., 2017b). Following treatment or mock treatment, the worms were then transferred to recovery plates, where they remained for another 24 hours.

Chronic intermittent ethanol treatment. Age-matched P0 (parental generation) worms were harvested during late L4 stage or early adulthood and divided into the control-line and ethanol-line. Treatment plates were prepared as described in the chronic EtOH treatment paradigm. The ethanol-line was placed on the EtOH-treatment plate and sealed with parafilm. In parallel, the control-line was placed on a mock treatment plate and then sealed with parafilm. Worms remained on the treatment plates for 15 minutes and were then transferred to recovery plates, where they remained for another 1 hour and 45 minutes. This treatment process was repeated three more times, resulting in four 15-minute treatments every two hours. After the final treatment, worms were moved to a final recovery plate for 1 hour and 45 minutes.

Transgenerational experimental design

After the recovery window in each treatment paradigm, the worms were transferred again to new plates for timed egg-laying to produce the age-matched F1 generation. Early F1 adults were allowed to lay eggs to produce the F2 generation before being collected for behavioral testing. This process was repeated using F2 early adults to produce the F3 generation. The F3 generation was raised to adulthood for behavioral testing.

Measuring acute intoxication

All worms used for behavioral testing were well-fed, age-matched early (day 1-2) adults. The individual animals selected for testing were those with generally healthy-looking, with no gross anatomical or locomotor defects.

Locomotor speed by centroid tracking. Methods used for recording and analyzing worm locomotor speed were modified from those previously described in (Bettinger, Leung, Bolling, Goldsmith, & Davies, 2012b). Movies were made on an Olympus SZX16 stereo microscope (magnification of 0.8x with a 0.5x objective) using a Flea2 camera (PointGrey imaging) and Streampix recording software. Ethanol-containing assay plates were prepared by dehydrating standard unseeded NGM plates prior to testing for 2 – 3.5 hours at 37° C with lids removed until plates reached a weight of 16.8 – 17.2 g (~ 2 – 3 g less than freshly made plates). Before being moved to assay plates, animals were transferred to an intermediate unseeded NGM clean-off plate to ensure no bacteria was also transferred onto the assay plate. In order to establish baseline speed, worms from the control-line and ethanol-line placed on the same unseeded NGM agar plate (containing no EtOH) within separate corrals made of a small copper rings (13 mm inner diameter), 10 worms/ring.

After acclimating to the baseline assay plate for approximately 2-3 minutes, worms were recorded 2 minutes at 2 frames per second with the lid of the assay plate removed during recording. The worms were then transferred onto an ethanol-containing plate, again placed in separate corrals on the same plate. After a 20-minute incubation on the parafilm-sealed plate, locomotion was recorded again for 2 minutes with lids removed during recording. Assaying worms from both lines on the same plate, or "yolking," allowed to control for small variations in plate condition. Distance traveled by each worm was measured by centroid tracking using ImagePro Plus (7.0) (MediaCybernetics) allowing us to calculate the speed (cm/m) of each worm and an average speed for each group of 10 animals was calculated. It should be noted that recording worms on EtOH required the lids to be off and each corral had to be recorded individually, leaving the second group exposed to open air for two minutes before being recorded. Because of this, the order in which the groups from each line were recorded was alternated (e.g. Control-line was recorded first during odd-numbered trials, EtOH-line recorded first during even-numbered trials).

Locomotor speed by measuring area covered. ImageJ software was used to re-analyze all videos. Although centroid tracking is generally accurate, automated tracking results in a certain amount of noise, likely generated by shadows and tiny movements of the worm, particularly when tracking intoxicated worms. These causes the software to track movement that is not actually occuring. We used area-measurement as a secondary analysis method to confirm accuracy.

Area covered is calculated by loading the video as a stack and then generating an "average" image of the stack, using the Z Project tool. Next, we subtracted the average image from

each slice of the stack to generated a new stack with background pixels removed. Using the Z Project tool on the new stack we generated a Z project image of the "Maximum" pixels of the stack. The maximum image was then thresholded using the IsoData auto threshold setting. This final image represents the tracks of the area covered by worms over the entire video, in white pixels on a black background. We calculated the percentage of total area covered by worms by measuring the total number of white pixels and dividing by the total area of the field of view. This value was also divided by the number of worms that participated in the video, generating the percentage of total area covered per worm. This process was repeated for all videos.

Statistical analysis

Baseline trials during which the average baseline speed was unusually low (>2 standard deviations below the mean) were excluded from analysis, along with the corresponding on-EtOH trial. Data that passed Shapiro-Wilk normality test were analyzed using standard t- or ANOVA tests and the Bonferroni or Sidak method for post hoc multiple comparisons tests (Zar, 1999). Data that did not pass the Shapiro-Wilk normality test were analyzed using the Mann-Whitney Rank Sum Test or Kruskal-Wallis ANOVA on ranks and Dunn's test for post hoc multiple comparisons (Zar, 1999).

RESULTS

Ancestral chronic exposure to EtOH may confer a very modest resistance to acute intoxication in subsequent generations

Because worms exhibit locomotor inhibition when exposed to EtOH in a dose-dependent manner (Davies, 2003), we used locomotion as a measure of EtOH sensitivity in F1 through F3 generations of EtOH-line and Control-line worms. The methods used for measuring locomotion during intoxication are modified from those described previously in Bettinger, 2012. Briefly, we measured the baseline speed by placing 10-15 worms from each lineage into separate copper ring corrals embedded on an unseeded NGM agar assay plate and recording locomotion for 2 minutes. The two groups of ~10 worms were then transferred to another assay plate containing 400 mM EtOH. After a 20-minute incubation on the EtOH-treatment plate locomotion of the worms was recorded once more for 2 minutes (Fig. 2.1). This exogenous concentration of 400mM EtOH (after a 20-minute exposure) results in an internal concentration of approximately 40mM by previous

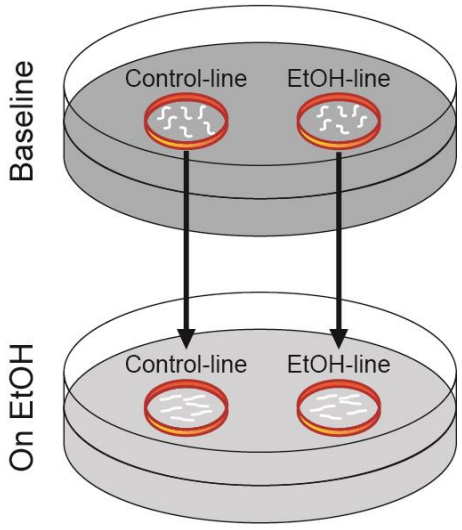


Fig 2.1 - Acute intoxication assay
This assay measures sensitivity to acute EtOH exposure by measuring locomotion. A trial consists of 10-15 worms from each line on the same plate in separate copper corrals, recorded for 2 minutes at baseline speed then moved to a plate containing 400mM EtOH (represented with lighter gray agar here) for the second part of the trial. After a 20-minute incubation on EtOH worms are recorded again for 2 minutes. The two groups that are tested together on the same baseline and EtOH-containing plates are considered paired or yoked.

estimates (Alaimo et al., 2012; Scott et al., 2017) This internal concentration is comparable to blood alcohol concentrations (BAC) observed during moderate intoxication in humans.

Two different measurement methods were used to analyze the locomotion of the worms after recording their behavior at 2 frames per second. First, we measure *average speed*, we used a semi-automated centroid tracking software, ImagePro Plus (7.0) (MediaCybernetics). This software automatically detects worms in the video and tracks the path of their centroid frame by frame over the entire two minutes. Using the total distance values generated by the software we calculated the average speed of each worm in centimeters per minute. The approach of the second method uses total two-dimensional area covered by the by the worms as a proxy for locomotion. In each cohort (a 3-generation population of worms generated from a single P0 treatment group), we generated 3-6 trials per generation. Each trial included 10-15 worms from each lineage, which were tested on baseline plates and on EtOH plates, meaning each trial generated 4 videos for analysis. A total of 9 cohorts were used, amounting to ~30 trials per generation.

Baseline locomotion, as measured by both average speed and area-covered, was comparable among both lineages (Fig 2.2 A, B). We had expected the EtOH-line worms to show less sensitivity to EtOH than the control-line. As with baseline locomotion, the on-EtOH measures were also not significantly different between lineages. Analysis of these raw baseline and on-EtOH locomotion measures did reveal a small effect of generation. Specifically, overall locomotion decreased with each generation, regardless of lineage or condition ($p = 0.0205$). This generational drift was unexpected, but may be due to some accumulation epigenetic variation over time. When working with *C. elegans*, worm strains

are kept in frozen stocks to minimize genetic drift over time. Each new cohort of worms tested were derived from a freshly thawed population of wild-type worms. Another

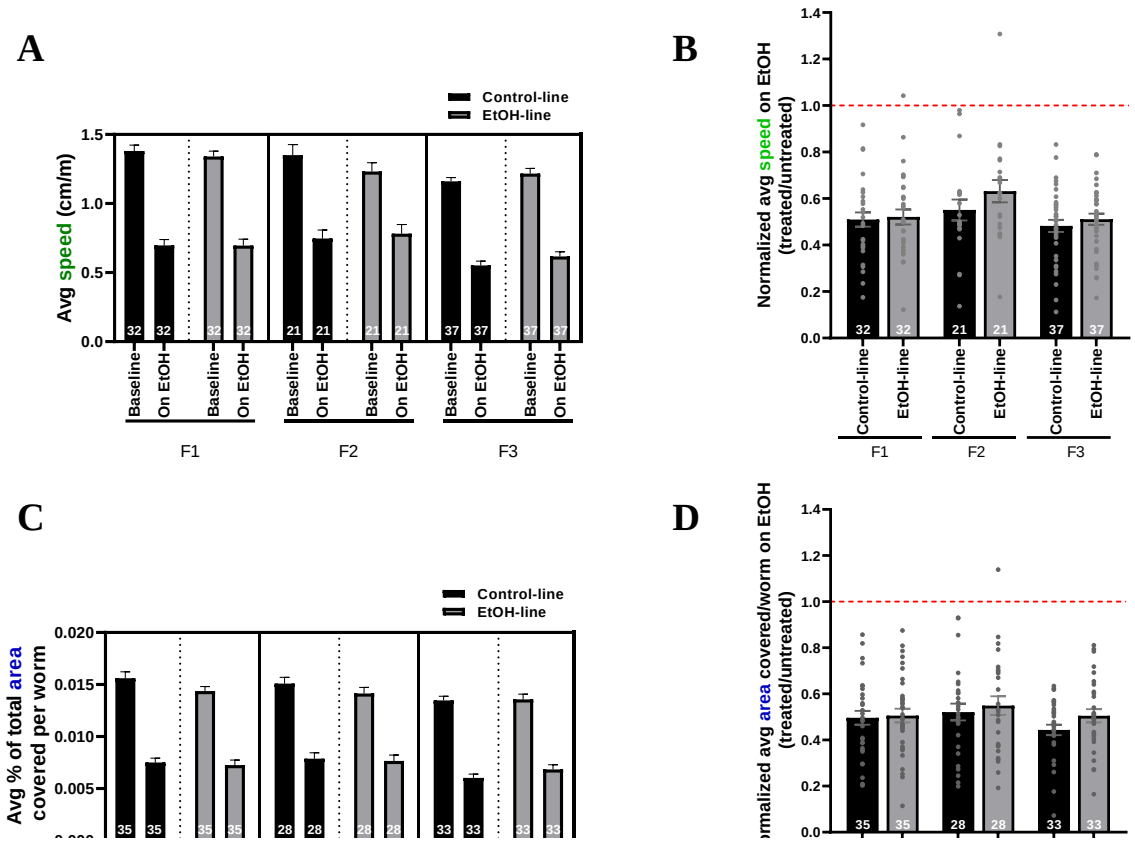


Fig 2.2 - EtOH-line animals show a trend toward ethanol resistance

Sensitivity to acute intoxication as measured by locomotion, comparing Control-line and EtOH-line animals. EtOH-line animals were derived from a P0 generation that underwent *chronic* ethanol treatment. ***A.*** Locomotion on baseline plates and EtOH-containing plates was assessed by using centroid-tracking software to measure the distance traveled over a 2-minute period and speed is shown in cm/m. Based on these measurements there was no significant difference either baseline speeds or intoxicated speeds due to lineage. ***C.*** Locomotion on baseline plates and EtOH-containing plates was assessed by measuring area of the field covered by worm tracks after 2 minutes as a percentage of the total area of the field. Because individual worm tracks are indistinguishable using this method, the percentage of area covered is divided by the number of worms in the field to calculate the average area covered per worm. Based on these measurements there was no significant difference either baseline speeds or intoxicated speeds due to lineage. ***B***, ***D.*** Intoxication as measured by locomotion on EtOH-containing plates relative to locomotion on baseline plates. ***B.*** Normalized average speed on EtOH in cm/m. ***D.*** Normalized average percentage of area covered per worm per minute while on EtOH. While the mean measures of locomotion in the EtOH-line were consistently higher than parallel control-line means, these differences do not appear to be significant. Number of trials indicated on each bar.

possible explanation may be inadvertent selection of less active individuals when propagating worms across generations. In order to control for differences in baseline locomotion, we converted trial averages of baseline and on-EtOH measures to a single normalized value. This was done by dividing the raw

on-EtOH trial averages by the corresponding raw baseline trial averages to generate a new on-EtOH value that represented as a fraction of the baseline average (Fig 2.2 *C* & *D*). For brevity, we will refer to the "normalized average speed on EtOH" as "normalized speed" throughout the rest of this chapter. Similarly, "normalized average percentage of total area covered per worm on EtOH" will be referred to as "normalized area." Analysis of these normalized values showed that there was no main effect of lineage on locomotion, but there was an effect of generation. Pair-wise comparison of lineage in each generation did not reveal a significant difference. When looking at the main effect of lineage (Table 2.1), we found that the EtOH-line overall has a slightly higher normalized average speed than control-line ($p = 0.14$). While not statistically significant, this may suggest a trend toward resistance in the EtOH-line compared to controls.

In our above analysis, normalized averages of all trials for control- and EtOH-lines are pooled together and compared. This does not take into account the groups that were paired together, or yoked, by trial. There is a considerable amount of variability between assay plates due to factors such as dehydrated weight, evaporation of EtOH, and lab conditions. We felt that comparing control-line and EtOH-line worms that were tested together may provide a more accurate representation of the effect of lineage on EtOH sensitivity. By taking the difference between the normalized trial averages of yoked pairs

of control-line and the EtOH-line, we generate a value we will refer to from here on as *relative EtOH-line sensitivity*. This is a single value that represents the normalized speed of the EtOH-line relative to the control-line, wherein the normalized speed of the control-line becomes 0.

Since it is not apparent which normalized trial average represent yoked pairs in Fig 2.2 *B* & *D*, these same values have been replotted in Fig 2.3 *A* & *D* with lines connecting averages that belong to yoked pairs. These to figures also serve to visualize the relationship between each pair. The relative EtOH-line sensitivity was calculated for both the speed and area measurement methods and presented in Fig 2.3 *B* & *C* and Fig 2.3 *E* & *F*.

To analyze the results of this approach we tested whether or not the relative EtOH-line sensitivity in each generation was significantly different than a hypothesized mean of zero. This would indicate whether or not the two lineages differed significantly in EtOH sensitivy when paired. Much like the unpaired normalized data, we saw very little difference between lineages with the yoked pairs. Again, we used both the speed and area-covered measures. In this case, based on the area-covered measures, in the F3 generation only, we found that the EtOH-line showed relative resistance to intoxication when compared to yoked control-line partners (Fig 2.3 *F* and Table 2.1).

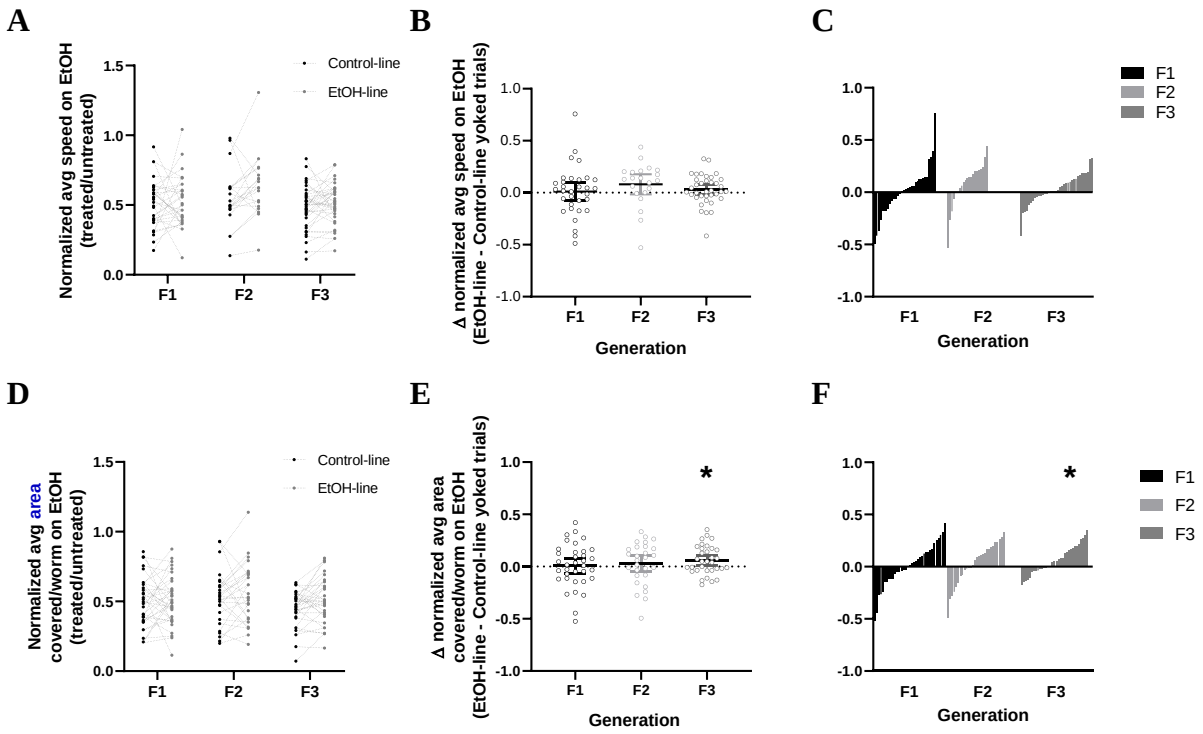


Fig 2.3 - Direct comparison of yoked lineage groups reveals similar trends toward resistance in the EtOH-line.

Differences between normalized average speed on EtOH (normalized speed) and normalized average percentage of total area cover/worm on EtOH (normalized area) for the EtOH-line and control-line yoked pairs in each set of trials. ***A, D***. Each point represents the normalized speed (***A***) or normalized area (***D***) for the control-line and EtOH-line of each trial. These values are the same as those shown overlaid on the bars in Fig 2.1B, ***D***. Dotted lines connect normalized values of control-line and EtOH-line groups tested on the same plate (yoked). ***B, C***. Differences between paired values shown in (***A***), [EtOH-line normalized speed – control-line normalized speed]. ***E, F***. Differences between paired values shown in (***B***), [EtOH-line normalized area – control-line normalized area]. ***B, E***. Differences represented as scatter dot plot grouped by generation. ***C, F.*** Differences represented as histogram of values in ascending order, grouped by generation. Error bars in (***B, E***) represent SEM.

Table 2.1 – Summary of acute intoxication analysis

Measurement method	Analysis of data method	Statistical test		p value	Significant?
Centroid tracking	Comparison of normalized intoxicated speeds	Two-way ANOVA	Main effect		
			Effect of Generation	* 0.0205	Yes
			Effect of Lineage	0.1401	No
			Multiple comparisons Conrol-line v EtOH-line		
			F1	0.9927	No
			F2	0.3658	No
			F3	0.8608	No
	Yoked trial difference	Test against hypothesized mean, μ = 0			
			F1	0.8009	No
			F2	0.0957	No
			F3	0.2304	No
Area covered	Comparison of normalized intoxicated speeds	Two-way ANOVA	Main effect		
			Effect of Generation	0.1659	No
			Effect of Lineage	0.1970	No
			Multiple comparisons Conrol-line v EtOH-line		
			F1	0.9941	No
			F2	0.9115	No
			F3	0.4000	No
	Yoked trial difference	Test against hypothesized mean, μ = 0			
			F1	0.7889	No
			F2	0.4716	No
			F3	*0.0153	Yes

After carrying out our transgenerational study using a chronic EtOH-treatment paradigm we wanted to investigate how varying the treatment schedule of the P0 generation would affect EtOH sensitivity in later generations. There are several variables in treatment schedule we suspect differentially modulate the transgenerational effects of EtOH, including developmental timing of treatment (eg. adolescent vs. adult), duration of treatment (acute vs. chronic) and frequency of treatment (single continuous treatment vs intermittent treatment). Therefore, for our second approach we chose investigate the transgenerational effects of intermittent EtOH-treatment on EtOH response. The treatment schedule differs from the chronic treatment in that, instead of a continuous 24-hour long exposure, worms are put on EtOH treatment plates for 15 minutes every two hours for a total of 4 treatments (Fig 2.4). Another important aspect of this treatment that differs from the chronic treatment is the effect on worm development during treatment. Chronic EtOH treatment, as we carry it out, starts at the late L4 stage (several hours before becoming adult) and continues for 24 hours. Because EtOH reduces feeding in worms by decreasing pharyngeal pumping rate, EtOH-line is effectively starved during this 24-hour period. Growth is also stunted as a result. This introduces two additional disparities between the EtOH-line and control-line. By limiting the amount of continuous time spent on EtOH the intermittent treatment reduces the impact of EtOH on feeding and growth.

Thus far we have only collected data from a several trials per generation using the intermittent treatment paradigm (N = 5-10 trials per generation). The main effect of lineage on normalized average speed was not quite significant as determined by two-way ANOVA ($F(1, 38) = 3.933$, $p = 0.0546$) and multiple comparison's using Sidak's multiple comparison test did not find significant differences between lineages in any generation.

However, the preliminary data reveal an interesting trend. Unlike with chronic treatment, normalized speed averages on EtOH tended to be lower in the EtOH-line than those of the

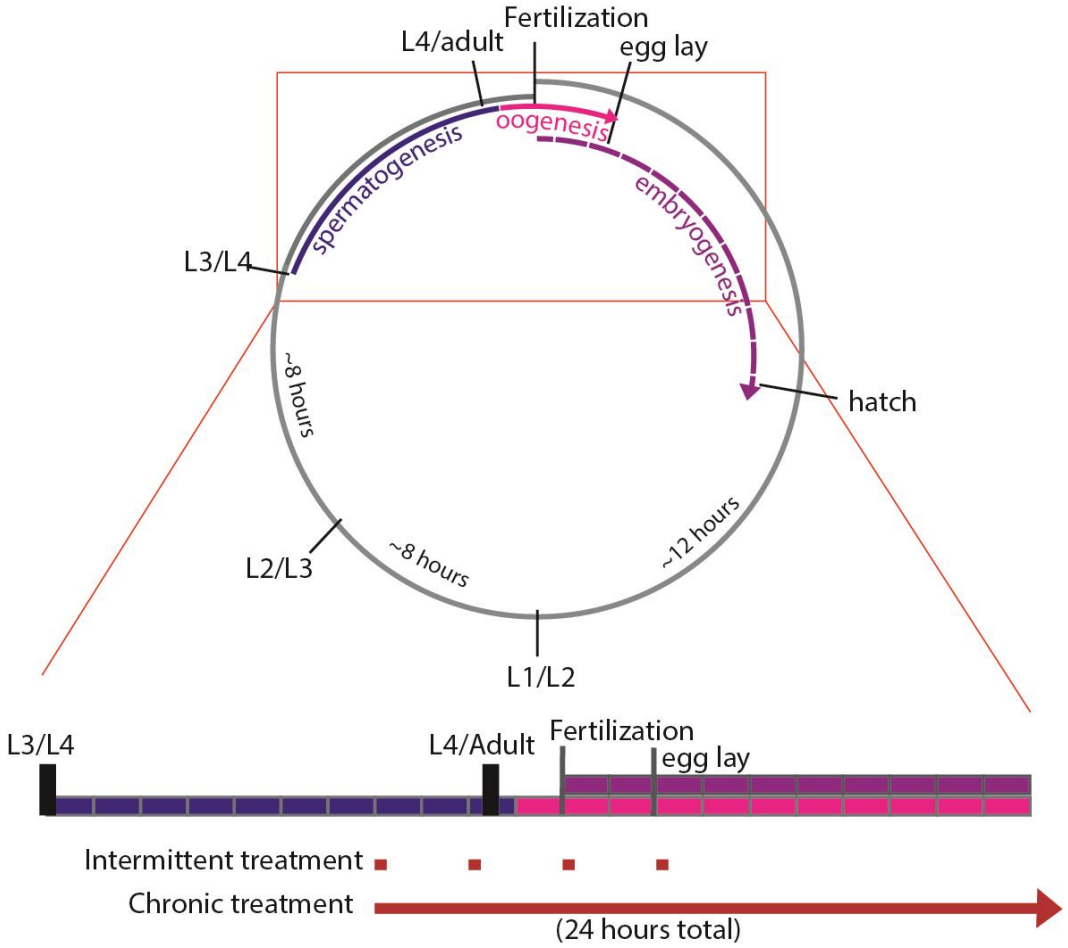


Fig 2.4 - *C. elegans* life cycle and EtOH-treatment timing
Life cycle of an individual worm runs along the gray broken circle (top) from fertilization to adulthood. Black dividing lines indicate developmental milestones and molts between larval stages. Inset (bottom) shows linear timeline (divided into 1-hour blocks) of development starting at L4 larval stage. Spermatogenesis (blue) occurs throughout the L4 larval stage and concludes just after the L4/adult molt. Oogenesis (pink) begins in early adulthood and continues throughout adulthood. Embryogenesis (purple) begins shortly after oogenesis. In both the chronic and intermittent EtOH treatment paradigms, EtOH was introduced at the mid- to late-L4 larval stage. Worms continue to be exposed to EtOH either intermittently or continuously for 8- or 24-hours, respectively.

control-line (Fig 2.5). Small sample size of these data may account for lack of significant differences between lineages. Still, the opposing directions of trends seen in between the two EtOH-treatment paradigms tested here is worth exploring further.

Finally, because of the variability in speed and area-covered measures between generations observed in the chronic treatment data, we wanted to investigate possible sources of inter-trial and inter-generation variability. One confounding variable we suspected as a contributing factor in this variability was seasonal condition. Noticeable seasonal fluctuations in lab temperature and humidity occur throughout the year. To test the effect of season on raw speed values, I compiled measures from all lineages and treatment groups. In Figure 2.5, average baseline speeds (*A, B*) and average speeds on EtOH (*C, D*) are grouped by season.

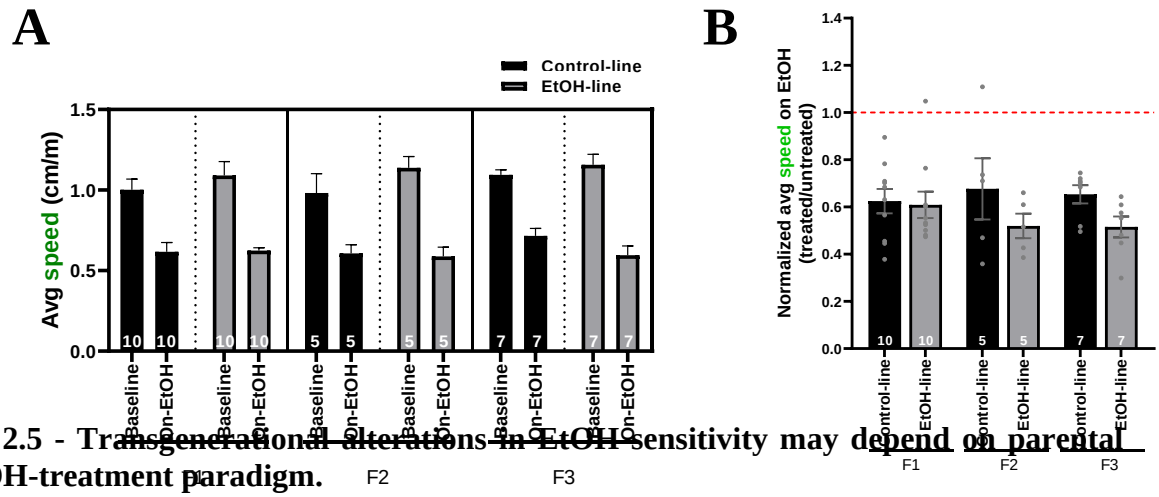


Fig 2.5 - Transgenerational alterations in EtOH sensitivity may depend on parental EtOH-treatment paradigm. Sensitivity to acute intoxication as measured by locomotion, comparing Control-line and EtOH-line animals. EtOH-line animals were derived from a P0 generation that underwent *intermittent* EtOH treatment. ***A.*** Locomotion on baseline plates and EtOH-containing plates in cm/m. ***B.*** Normalized average speed on EtOH in cm/min.

It is clear that season has a strong effect on baseline speed in both conditions (one-way ANOVA, F (2, 259) = 46.95 P<0.0001). In particular, large differences were observed when comparing spring vs. summer (P<0.0001, Tukey's HSD) and spring vs. winter

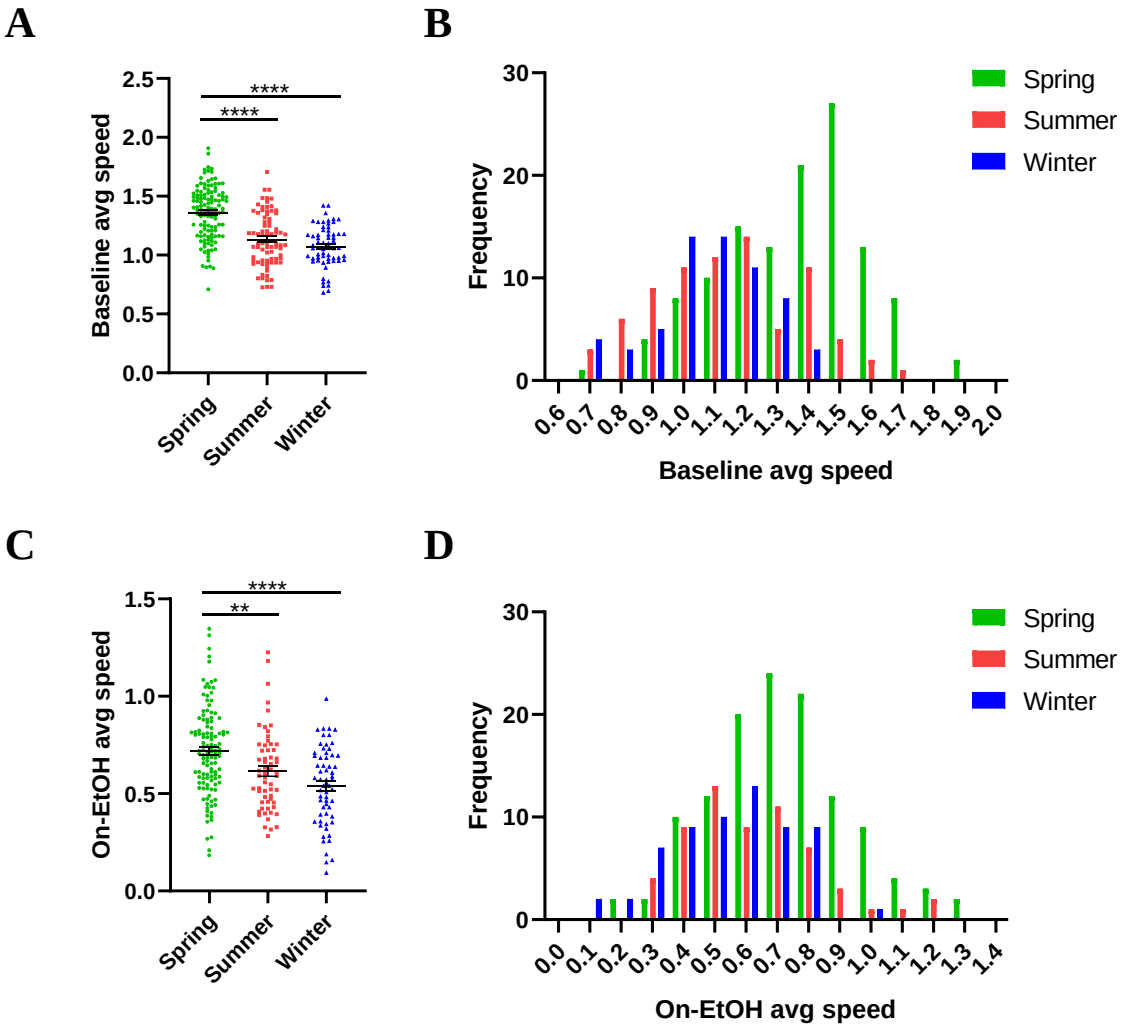


Fig 2.6 - Seasonal changes significantly affect speed in both conditions.
A, C. Plot of trial averages for baseline (*A*) and on-EtOH (*C*) conditions, pooled from all lineages and treatment paradigms tested, grouped by season. Each point represents the average speed of a single trial. Error bars represent SEM. Season had a strong effect on average speed in both conditions. ***B, D.*** Interleaved histograms of baseline average speeds grouped by season (*B*) and average speed on EtOH grouped by season (*D*).

($P<0.0001$, Tukey's HSD). There was not a significant between summer and winter, however ($P=0.220$, Tukey's HSD).

Season also had a large effect on average on-EtOH speed (one-way ANOVA, $F(2, 241) = 15.46$, $P<0.0001$). As with the baseline average speed, there was a highly significant difference between spring and winter ($P<0.0001$, Tukey's HSD), but the difference between spring and summer was not as pronounced ($P = 0.0062$, Tukey's HSD).

Seasonal conditions had similar effects on both baseline average speed and speed on EtOH. The main measure we were concerned with in this study was the normalized average speed on EtOH, which is a ratio of other two averages. With that in mind, if seasonal changes have the same effect in both conditions this should not affect our normalized averages. However, the seasons appear to affect baseline and on-EtOH average speed to a different degree. The smaller difference between spring and other seasons we saw for on-EtOH average speed may be due, in part, to a floor effect. Further investigation into seasonal variability on average speeds is needed to determine the degree to which this is a concern. Regardless, minimizing variability of lab conditions is clearly a priority for future studies of this nature.

DISCUSSION

Here we present the first study, to our knowledge, that examines the effects of parental chronic ethanol exposure in the subsequent F1-F3 generations. Although a similar study was carried out in rats (testing F1-F3, also), the parental exposure was limited to only a brief prenatal treatment (Nizhnikov et al., 2016). In our approach, the parental chronic EtOH treatment, as well as recovery from EtOH, takes place prior to conception of the embryos that give rise to the F1-F3 individuals later tested. Thus, the findings from our treatment paradigm are unique in that they are the result of parental somatic cell and germline cell exposure only. This represents a valuable approach because any epigenetic alterations transferred to the progeny would have resulted from some mechanism that persisted from the time of EtOH treatment to post-conception.

In this study, we first tested our hypothesis that chronic treatment with EtOH in the parental (P0) generation would lead to a resistance to acute EtOH intoxication in subsequent generations. Our results did not reveal a significant difference in sensitivity to acute intoxication between the control line and the EtOH line in any generation, although there was a modest trend toward resistance in each generation. When we repeated this transgenerational study using an intermittent EtOH-treatment schedule in the P0 generation we also did not see a significant difference between the control line and EtOH line in any generation. The intermittent treatment results did differ from the chronic treatment, however, in that there was an opposite trend; the intermittent treatment group showed a modest trend toward increased *sensitivity* to acute intoxication in the EtOH line, relative to

the control line. Although these results were not sufficient to support our hypothesis of resistance to acute intoxication in the EtOH-line, this is probably not the end of the story. Transgenerational epigenetic inheritance can be influenced by any number of factors, and not necessarily our experimental treatment alone (Bohacek & Mansuy, 2012; Heard & Martienssen, 2014; Rankin, 2015; F. M. Vassoler & Sadri-Vakili, 2014). Even in our relatively well-controlled laboratory setting, variables beyond parental EtOH-treatment likely influenced transgenerational changes in behavior and gene expression. These include starvation, treatment schedule and seasonal conditions, on which I elaborate on below.

An unintended consequence of our chronic EtOH-treatment paradigm is starvation. The intoxicating dose of EtOH reduces the worm's rate of feeding by directly inhibiting pumping of its pharynx, a bi-lobed pump that sucks in bacteria as food via peristalsis. In turn, this dramatically decreases food intake. We began the chronic treatment during the L4 larval stage of development. During this time period the worm normally grows by 25% in length and around 40% in volume as it enters the adult stage (Byerly, Scherer, & Russell, 1976; Uppaluri & Brangwynne, 2015). Although we have not quantified it, growth was stunted in the EtOH-line P0 worms following chronic EtOH treatment. A recent worm study investigated the intergenerational effect of maternal dietary restriction (DR) by raising DR worms in liquid culture containing low concentrations of the *E. coli* food source. This dietary restriction results in smaller early brood sizes but individual progeny were larger and were later resistant to the effects of starvation (Hibshman, Hung, & Baugh, 2016). Another recent worm study invested the transgenerational effects of starvation. In that study, the P0 generation was exposed to prolonged starvation (8 days) during the L1

larval stage, which leads to “larval arrest.” When the P0 were allowed to recover from starvation, they took longer to grow to adulthood, had reduced adult size and reduced fecundity (Jobson et al., 2015). Progeny of these starved P0 also had reduced embryo quality, small brood size, and their progeny were smaller, in contrast with the study described above. Based on our casual observations (never quantified), our P0 EtOH-line may have had smaller brood sizes as well. This will need to be quantified in the future. We did not note a difference in F1 size, but measurements of F1 individuals could be taken from videos of acute intoxication assay. Taking all of this evidence together, there is reason to believe that starvation produces transgenerational effects that may have interfered with our transgenerational EtOH-treatment results. In the future, the effects of starvation alone on P0 worms and their progeny needs to be examined by running a starved control line alongside the non-starved control line and EtOH line.

In addition to our chronic EtOH-treatment study, we investigated the transgenerational effects of intermittent EtOH-treatment. As with our chronic EtOH-treatment, we expected this treatment to result in resistance to acute intoxication in subsequent generations. Also, if starvation did interfere with the results of the chronic EtOH-treatment study, this approach should have reduced that interference. In contrast to our findings for chronic EtOH treatment, the effect lineage trends toward increased *sensitivity* in the EtOH-line. These unexpected results warrant further investigation.

After completing these two studies we discovered another variable that we had not considered, which may have interfered with our results: seasonal conditions. When average trial speeds (for all trials conducted throughout these studies) were sorted by season, we

discovered a significant effect. Specifically, average trial speeds from spring months were higher than those of either winter or summer months. This was true of baseline trial speeds as well as the trial speeds while on EtOH. Seasonal changes, along with corresponding changes in the indoor heating and cooling systems, lead to fluctuations in both ambient temperature and humidity in the laboratory. These two factors could easily affect worm behavior, either through their impact on the NGM plates the worms are raised and tested on, or through direct sensory input to the worm, as they are able to sense even 0.1°C changes in temperature (Ramot, MacInnis, & Goodman, 2008). Humid conditions also increase the likelihood of mold contamination on NGM plates. Certain molds are pathogenic toward *C. elegans* and its presence is likely to affect worm behavior (Schulenburg & Félix, 2017).

It is rare in *C. elegans* literature to see mention of seasonal conditions contributing to variability in behavior, although the methods often indicate that worms raised at 20°C with no indication of temperature fluctuations. Guidelines for conducting behavioral experiments do, however, recommend recording ambient condition information for consideration during data analysis and troubleshooting (Hart, 2006). One study, in which researchers generated a behavioral database of a large number of mutant strains, concluded that season did have a significant effect on various behaviors (foraging, forward speed, reversal frequency, and exploratory range) based on a significant effect of the month of testing (Yemini, Jucikas, Grundy, Brown, & Schafer, 2013). This effect was not investigated further, but the authors accounted for this variability by comparing mutant data to wild-type worms tested within a three-week window of the mutants. In another

recent study, humidity was also found to reduce robustness of magnetotaxis (orientation to and navigation by the magnetic field) in *C. elegans* (Bainbridge et al., 2019). To avoid significant effects on behavior by humidity and temperature fluctuations, it is suggested that researchers should avoid testing the worms during periods of high humidity, rain, and large temperature fluctuations (Bainbridge et al., 2019; Goodman, 2014). When conducting multigenerational studies such as this one, which sometimes bridge different seasons, it may be difficult to plan around weather conditions. Still, temperature, humidity and weather conditions should be recorded daily and experiments should be avoided during seasonal transitions. Use of incubators during these experiments may be helpful in mitigating these effects in the future, as would conducting the experiments in an available space that is less affected by weather conditions.

Besides investigating heritable phenotypes that result from ancestral EtOH-treatment, gene expression changes also need to be surveyed. We are particularly interested in looking at changes in *slo-1* expression. Through two saturating forward genetic screens, the *slo-1* gene found to be a major target for EtOH is required for acute intoxication in *C. elegans* (Davies et al., 2003). This gene codes for the BK channel, a large-conductance calcium- and voltage-activated potassium channel. Worms lacking this channel show very strong resistance to intoxication, show more severe EtOH withdrawal-related impairments (Scott et al., 2017b), and it is a conserved target for EtOH that is also found in mouse, fly and humans (Mulholland et al. 2009; Treistman and Martin 2009; Bettinger and Davies 2014). The resistance to intoxication of worms lacking this channel make *slo-1* an appealing candidate gene to investigate in worms following ancestral EtOH treatment. A

broader survey of gene expression changes following parental EtOH-treatment may inform future endeavors in investigating behavioral transgenerational effects of EtOH.

Most of the transgenerational studies using *C. elegans* involve using exogenous double stranded RNA (RNAi) as the "trigger" to attempt to induce a transgenerational effect. These studies have been immensely valuable in understanding how epigenetic programming can be transmitted to progeny and across generations. However, despite the great deal of overlap between the mechanisms involved in RNAi and those used by endogenous small interfering RNAs (endo-siRNAs), RNAi triggers do not necessarily reflect how the biological response of the animal to environmental triggers can produce heritable transmission of epigenetic information. Therefore, it is imperative that environmental triggers are employed in transgenerational studies in order to understand how and when TEI can be "naturally" induced in *C. elegans*, as opposed to using the "artificial" trigger of RNAi. Our study utilizes alcohol exposure specifically as a potential trigger for TEI in *C. elegans*, but several others have also been investigated recently.

There are several recent studies using *C. elegans* that utilize environmental triggers in their approach to study TEI. As described above, starvation and dietary restriction have been used as an environmental trigger for TEI (Hibshman et al., 2016; Jobson et al., 2015). Both of these studies uncovered effects of starvation or dietary restriction that involved, growth, brood size, and stress resistance phenotypes. Other environmental triggers that have been investigated in *C. elegans* are pathogenic bacteria exposure (Moore, Kaletsky, & Murphy, 2019) and exposure to diesel particulate matter, an industrial air pollutant (M. Wang et al., 2019). In the former, it was found that when worms learned to avoid

pathogenic bacteria Pseudomonas aeruginosa (PA14) in the P0 generation, progeny that had never encountered the bacteria showed avoidance for four generations (F1-F4). Inheritance of this pathogenic avoidance phenotype could be prevented by disruption of the piRNA pathway.

The recent study that investigated the transgenerational effects of diesel particulate matter (DPM) found that a 24-hour exposure to 1.0 µg/mL DPM in the parental (P0) generation during the L4 larval stage resulted in increased germ cell apoptosis in these animals, as well as reduced brood size. Germ cell apoptosis was assessed in the F1-F5 generations following P0 exposure. A less pronounced increase in germ cell apoptosis was seen in the F1 generation, but by the F2 generation, the number of apoptotic germ cells had recovered to the level seen in control (unexposed) animals. The increase in apoptotic germ cells in F1 did not occur when P0 was exposed to a lower concentration (0.1 µg/mL) of DPM. The researchers also carried out another transgenerational study in which worms were exposed to DPM in consecutive generations, which they referred to as "continuous" exposure. In the continuous exposure experiments, each generation, P0 and F1-F5, was exposed to DPM for 24 hours during the L4 larval stage. This resulted in a similar increase in apoptotic germ cells in the adults of each generation, with no difference between generations. The continuous exposure did, however, lead to a gradual decrease in brood size starting in the parental generation and continuing to decrease each generation through to F5. Unlike the pathogenic bacteria avoidance study, which provided strong evidence for true transgenerational epigenetic inheritance by an environmental trigger, these results more likely demonstrate a case of consecutive intergenerational effects. If DPM exposure

leads to reduced brood size and increased germline apoptosis in the exposed generation and immediate progeny, it is feasible that consecutive or continuous exposures would have an additive effect, wherein the toxin leads to reproductive decline across generations.

Although the environmental triggers used to induce TEI differ between these studies and also differ from our own, there are some commonalities to the approaches used. For example, in several of the studies, exposure to the trigger took place during a developmental window in the parental generation that may be vulnerable to epigenetic perturbation. Pathogenic bacteria avoidance training was carried out during the L4 larval stage, as well as DPM exposure and our EtOH treatment. The *C. elegans* germline is developing during this stage, and importantly, spermatogenesis takes place and is completed just as the worm transitions from L4 to young adult. This means that when a trigger is initiated during the L4 stage and persists for 24 hours (as was the case in all three of these studies), the entire lifetime supply of sperm cells the worm carries would have been exposed to the trigger. Besides the developmental timing (i.e. exposure during L4 stage), the duration of the trigger may be a key factor in TEI. This time window and 24-hour duration of exposure successfully induced transgenerational avoidance of pathogenic bacteria, but the researchers did not see inheritance of this behavior with a shorter (4-hour) avoidance training in the parental generation. This timing and duration of the parental trigger also led to intergenerational inheritance of reproductive impairment following DPM exposure. Although we did not see a significant heritable phenotype in our study using this timing and duration of treatment, these two studies suggest it is a promising approach for transgenerational research in the worm.

ACKNOWLEDGEMENTS

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CHAPTER THREE: FORWARD GENETIC SCREEN YIELDS RESCUE OF PARKINSONIAN MOTOR DEFECT IN DOPAMINE DEFICIENT *C. ELEGANS* MUTANT

ABSTRACT

Dopamine is a conserved neuromodulator used to maintain and switch between motor patterns across animals as diverse as worms, flies, mice, and humans. Although boosting residual dopamine signaling in Parkinson's disease (PD) patients as well as animal models of PD represents an effective treatment to preserve motor function, treatments to maintain function once dopamine neurons fully degenerate are lacking. To find novel approaches to maintain movement in the face of negligible dopamine, we performed a genetic screen in *C. elegans* to identify mutations that suppress the motor transition deficit in the mutant *cat-2*. This mutant lacks the major dopamine synthetic enzyme tyrosine hydroxylase and fails to transition smoothly from swimming to crawling motor patterns. We identified four *cat-2* suppressor (*ctsp)* mutants that transitioned efficiently at rates similar to or faster than wild-type worms. Identifying conserved genes that can be mutated to preserve motor function in the absence of dopamine may reveal insight into repair of dopamine-deficient circuitry in higher animals.

INTRODUCTION

The loss of dopamine-secreting neurons within the basal ganglia causes the motor defects observed in Parkinson's disease. Most medical treatments for Parkinson's disease (PD) rely on boosting or altering the function of residual dopamine neurons in the substantia nigra. Two of the most effective approaches used in treating Parkinson's disease – the medication levodopa and deep brain stimulation – can dramatically improve symptoms in some patients. However, long-term use and increased dosage of levodopa is known to exacerbate motor symptoms and cause dyskinesia. Furthermore, a subset of symptoms that are typical in late-stage PD are resistant to both of these popular treatments. Thus, there is a critical need to discover alternate approaches to maintain motor function in late-stage PD.

Animal models of PD have helped usher in new treatments and a better understanding of underlying neuronal dysfunction. Studies in both vertebrate and invertebrate species have found that dopamine plays a critical role in modulating motor output. Numerous studies using vertebrate models – primarily rodents and non-human primates – have utilized dopaminergic neurotoxins to induce lesions in nigrostriatal dopaminergic neurons. These lesions lead to parkinsonian and similar motor defects (Schober, 2004). Using pharmacological and optogenetic manipulations, these same

models have been essential in uncovering the specific roles of the D_1- and D_2- like receptors and the pathways they modulate in the striatum (Kravitz et al., 2010). Invertebrates, including the nematode *Caenorhabditis elegans*, also use dopamine as a key modulator of motor activity (Hills, Brockie, & Maricq, 2004; Omura, Clark, Samuel, & Horvitz, 2012; Sawin, Ranganathan, & Horvitz, 2000). *C. elegans* offers unique advantages in studying the neuromolecular basis for motor pattern transitions. It can withstand genetic manipulations many other models cannot. Whereas flies and mice cannot develop to adulthood without dopamine, *C. elegans* is viable and grossly normal even after deletion of the dopamine synthetic enzyme tyrosine hydroxylase (Sulston, Dew, & Brenner, 1975; Zhou & Palmiter, 1995; Zhou, Quaife, & Palmiter, 1995). *C. elegans* also is tiny (1 mm), has very short generation times of 3 days, and produces hundreds of genetically identical progeny in a matter of days (Brenner, 1974). These attributes allow forward genetic screening for mutations that favor motor phenotypes. Our lab previously demonstrated that *C. elegans* traverses its environment using two distinct forms of motion: swimming and crawling (Pierce-Shimomura et al., 2008; A. Vidal-Gadea et al., 2011). Moreover, disruption of dopamine signaling in worms produces defective switching of motor patterns, analogous to the gait switching and motor pattern initiation deficits caused by PD (Vidal-Gadea et al., 2011). Specifically, disrupting dopaminergic pathways in *C. elegans* – either through ablation of dopamine neurons, knocking out specific genes involved in dopamine synthesis or D1-like dopamine receptor signaling – causes worms to freeze their posture for a brief period when switching from swimming to crawling forward (Vidal-Gadea et al., 2011).

One of the mutants that displays this deficit is the *cat-2* mutant, which lacks tyrosine hydroxylase, the major biosynthetic enzyme for L-Dopa, a precursor for dopamine (Lints & Emmons, 1999; A. Vidal-Gadea et al., 2011). Without this enzyme, this mutant produces little to no dopamine (Hardaway et al., 2012). Here, we set out to uncover novel mechanisms that can repair the swim-to-crawl transition in this dopamine-deficient mutant by carrying out a forward genetic screen. From this screen, we isolated four suppressor mutants that transition from swimming to crawling as fast or faster than wild type. We went on to characterize these mutants by evaluating dopamine-mediated behavioral responses.

MATERIALS AND METHODS

Worm husbandry

All worms were maintained as previously described (Brenner, 1974). Briefly, worms were raised at 20°C on standard plates which are 6-cm-diameter Petri dishes filled with 12 ml of nematode growth media (NGM) agar seeded with OP50 bacteria. The strains used in this study were N2 (Bristol), *cat-2 (tm2261).* Suppressor mutants generated from our forward genetic screen were assigned strain names using the designated strain prefix used by our laboratory (JPS) and were also assigned unique allele name using the designated allele prefix used by our laboratory (*vx*): JPS871 *vx26*, JPS872 *vx27*, JPS872 *vx28*, and JPS896 *vx29*. Whenever possible, worms were propagated by harvesting age-synchronized embryos. This was done by using either timed egg-laying or by treatment of gravid adults with a bleaching solution (20% sodium hypochlorite/10% 5 N NaOH).

Forward genetic screen

Mutagenesis with ENU and propagation: We chose to use the *cat-2* mutant strain carrying the predicted null allele *tm2261* because it deletes a 211 bp segment of the gene, replaced by a 3 bp insertion, and were thus unlikely to recover intragenic revertant mutants from our suppressor screen. *cat-2* mutants were mutagenized by incubating a synchronized population of L4-stage individuals in ENU for four hours. Following incubation, the worms were thoroughly rinsed and placed onto a new NGM agar plate seeded with OP50 to

recover for 3 hours. After recovery, 100 of these mutagenized individuals (the P0 generation), were picked and distributed amongst 10 plates (10 worms/plate). These P0 worms were allowed to lay F1 eggs for a 12-hour period before being moved to new plates to lay the next clutch of F1 eggs, again, for a 12-hour period. A total of 5 clutches of F1 offspring were generated, spread over 50 plates. When these F1 worms reached adulthood, they were allowed to lay eggs (F2) for several hours before the adults were removed from the plates, leaving behind only the F2 eggs. When the F2 generation reached adulthood they were screened (as a population) for swim-to-crawl transition efficiency (described below).

Screening for suppression of the cat-2 swim-to-crawl defective phenotype: Day-one, adult F2 worms were screened as a population, alongside those laid and raised on the same plate. The adults from each plate were prepared in the same fashion as in the radial dispersion assay, described below. Briefly, they were transferred to an Eppendorf tube filled with NGM buffer, rinsed twice, and after removing most of the supernatant they were transferred (suspended in NGM buffer) to the center of a radial dispersion assay plate with diacetyl. However, instead of recording the dispersion, a 1.5-minute timer was initiated as the first worm emerged from the puddle. Individual worms that reached the outermost zone of the plate at the end of this 1.5-minute interval were isolated in order to generate F3 progeny. Individuals isolated from these screening trials were considered potential suppressor mutants for the *cat-2* swim-to-crawl defective phenotype. Offspring of these individuals were retested in order to confirm that this suppression persisted in the F3

generation. Out of 20 potential suppressor lines generated from F2 individuals isolated in the initial screening step, only 5 maintained suppression of the swim-to-crawl defective phenotype in F3 generation and beyond. One of these 5 failed to produce viable offspring, leaving 4 suppressor strains to further characterize.

Behavioral assays

Radial dispersion : Locomotor behavior during the swim-to-crawl transition was assessed by modifying previous methods (Vidal-Gadea et al., 2011) The plates used for this assay were 6-cm diameter plate filled with 12 mL of NGM agar and we selected plates that had a smooth, unblemished surface on the agar, free of any bubbles. These plates were prepared by first marking the precise center on the bottom of the plate and then by placing a solution of diacetyl (1 µl diacetyl: 1000 µl of 200 proof EtOH) along the perimeter of the plate to motivate the worms to migrate outward from the center of the plate. The diacetyl was spread along the outermost edge of the agar by adding a four 2 µl droplets of the solution at the outermost edge of the agar on the plate - one drop in each quadrant, equally spaced apart – and then using a thin watercolor paintbrush to quickly spread the diacetyl around the perimeter by gently pressing the paintbrush against the outer edge of the agar and steadily rotating the plate for 2-3 revolutions.

All strains were tested on the first or second day of adulthood. Age-matched adult worms, in groups of between 50 and 300 individuals per trial, were transferred from NGM agar seeded plates into an Eppendorf tube containing 1.5 ml of liquid NGM buffer. To remove any residual OP50 bacteria that may have been transferred along with the worms,

they were then rinsed by allowing all worms to settle to the bottom of the tube, removing the supernatant, and refilling the Eppendorf tube with liquid NGM buffer up to the original 1.5 ml volume. This process was repeated for a total of two rinses. The supernatant was then removed once more, so that the worms were suspended in a minimal volume of the buffer. Using a Drummond pipette fitted with a 25 µl glass capillary tube, the worms (suspended in buffer) were drawn up into the tip of the capillary tube and transferred to an assay plate by ejecting a small puddle of the worm/buffer suspension onto the precise center of the plate.

Once the puddle was in place, the assay plate was placed inside of a darkened recording chamber on a stage using darkfield-style illumination. The full area of the plate was recorded, using a simple USB microscope (Plugable 250x Digital USB Microscope), beginning just prior to the emergence of the first worms from the puddle. Recording continued for a total of 10 minutes at a rate of 1 frame every 2 seconds using Plugable Digital Viewer software.

Video analysis: Raw video files were processed using ImageJ. From each dispersion trial, a two-minute (60 frame) segment was isolated, beginning with the first frame showing one or more individuals having emerged from the liquid. The plate area was then divided into zones by drawing 3 concentric circles with an area 25, 50, and 75 percent of the entire plate area, respectively. After overlaying each frame with these target-like zone divisions, the number of worms in each were tallied and recorded. These counts were taken at 20 second intervals between time 0 (emerging from the liquid) to 120 seconds later.

Characterizing suppressor strains: In the following behavioral assays, day 1 or day 2 adults were used. Suppressor mutants were tested alongside a wild-type control group. Trials during which the wild-type control did not perform as expected were excluded.

"Dry" radial dispersion: Plates were prepared in the same manner as in the original radial dispersion assay, described above. In these dry radial dispersion assays, however, worms were transferred directly to the center of the testing plates, instead of being transferred to the center in a droplet of NGM buffer. In each trial, a group of fifteen worms with gathered onto a pick (transfer tool) and placed carefully in the center of the plate. A timer was started once the worms made contact with the agar surface. The plate was then placed inside of the recorder chamber. The video recording was initiated at 20 seconds after the worms were placed on the agar. Worms were recorded for 1 minute and 40 seconds (ending at 2 minutes after the worms were placed). Video analysis was performed as described above.

Radial dispersion with exogenous dopamine treatment: Preparation for radial dispersion was carried out as described above, up to and including the two rinses with NGM. After the second rinse, when all worms had settled to the bottom of the Eppendorf tube, enough supernatant was removed to leave a volume of 100ul in the tube (NGM + worms). We then added 100 µl of a 50 mM dopamine solution in NGM to the tube, leaving the worms suspended in a dopamine solution with a final concentration on 25 mM. The worms remained in the dopamine solution, undisturbed, for 5 minutes, after which we removed the supernatant, leaving the worms in a minimal volume of the solution for transferring. Using a Drummond pipette fitted with a 25 µl glass capillary tube, the worms (suspended

in the remaining dopamine/NGM solution) were drawn up into the tip of the capillary tube and transferred to an assay plate by ejecting a small puddle (~5ul) of the worm/solution suspension onto the precise center of the plate. The worms were then recorded as described in the radial dispersion methods above.

Treatment with 6-OHDA: To induce dopamine neuron degeneration, we adapted previously established protocols which utilize the toxin 6-hydroxydopamine (6-OHDA) (Nass, Hall, Miller, & Blakely, 2002; Tucci, Harrington, Caldwell, & Caldwell, 2011). Because of its tendency to oxidize quickly, the 6-OHDA solution used for treatment is made with ascorbic acid and was always freshly prepared shortly before use.

In preparation for 6-OHDA treatment, worms from each strain to be tested were age-synchronized using timed egg-laying. When these embryos reached the L4 larval stage they were harvested for treatment by transferring from their home plate to an Eppendorf tube with dH_2O in and rinsing 2 to 3 times with dH_2O, in a similar fashion to the NGM rinses used in preparation for radial dispersion. After the final rinse, when the larvae had settled to the bottom of the Eppendorf tube, enough supernatant was removed to leave a volume of 250 µl in the tube (dH_2O + worms). Next, 250 µl of a 60 mM 6-OHDA/80 mM ascorbic acid stock solution was added to the tube. This left the larvae soaking in a working solution of 30 mM 6-OHDA/40 mM ascorbic acid. The tube was placed in a tube rotator for 30 minutes at room temperature. The L4 larvae were then removed from the tube, washed 4 times with dH2O, and transferred to a seeded plate for recovery. After 24 hours, 50 adult worms from each strain were selected and tested using the radial dispersion assay, as

described above. Any worms showing severely impaired mobility while on the recovery plate were excluded from testing.

Statistical analysis

Data that passed Shapiro-Wilk normality test were analyzed using standard t- or ANOVA tests and the Bonferroni or Sidak method for post hoc multiple comparisons tests (Zar, 1999). Data that did not pass the Shapiro-Wilk normality test were analyzed using the Mann-Whitney Rank Sum Test or Kruskal-Wallis ANOVA on ranks and Dunn's test for post hoc multiple comparisons (Zar, 1999).

RESULTS

Dopamine-deficiency impairs efficient swim-to-crawl transition

We previously demonstrated that D1-like dopaminergic signaling was required for *C. elegans* to efficiently transition from swimming to crawling forms of motion (A. Vidal-Gadea et al., 2011). In this earlier study, we quantified the efficiency of crawl initiation by video recording single worms as they moved out of a small puddle of water one at a time. Although sufficient to demonstrate the importance of dopamine signaling in motor transition, this single-animal analysis could not analyze populations of worms.

To quantify the efficiency of switching from swimming to crawling for a population of worms, we developed a new assay (Fig. 3.1 *A* and *B*) in which the worms were video recorded as they dispersed from the center of an assay plate after emerging from a puddle. The assay plate was divided into 4 zones (Start, zone 1, zone 2, zone 3), demarcated by concentric circles with diameters that measure 0.75, 0.50 and 0.25 of the full diameter of the plate. A small population of worms (n = ~30-55) was rinsed before being placed in a puddle (10 µL) at the center of an agar-filled plate, in the starting zone. Worms are unable to escape the puddle until it is almost fully absorbed into the agar because they cannot overcome the surface tension. Wild-type worms continued swimming in the puddle until it was nearly completely absorbed into the agar. At this point, the worms gained traction with the agar surface and initiated crawling. Most worms then crawled to reach the volatile butter-smelling chemoattractant, diacetyl, which was placed around the edge of the plate (zone 3) immediately before the assay. The efficiency of swim-to-crawl transition could be visualized quantitatively as to how the distribution of worms expands from the center

radially as a function of time. Worms start out distributed completely in the most central area of the plate, but soon migrate toward the edge of the plate during this radial dispersion assay.

To test the requirement of dopamine for efficient crawl initiation in our new assay, we compared how the *cat-2* mutant performed versus wild type. The *cat-2* mutant lacks tyrosine hydroxylase which is the conserved rate-limiting enzyme used to synthesize dopamine in worms, mice, and humans (Lints & Emmons, 1999; Nass, Miller, & Blakely, 2001). We found that while wild-type worms migrated at a steady pace toward the outer zones, the *cat-2* mutants remained stagnate in the inner zones for much of the assay (Fig 3.1). Displacement from the origin was determined by tallying the number of worms in each zone at regular intervals and converting these counts to a fraction or percentage of the total number of worms in that trial. On average, 2 minutes was sufficient for just over half of wild-type worms to migrate beyond the inner zones (start & zone 1) of the plate and reach the reach the outer zones (Fig 3.1 *E*; 35.6% ± 10.2, 15.01% ± 13.2 in zones 2 and 3, respectively). The efficiency of the migration by wild-type worms is illustrated in Figure 3.1 *C*, which shows the distribution of the percentage of worms in each zone at each 20 second interval. Here we see that the peak of the distribution of wild-type worms shifts steadily from starting zone (origin) toward zone 2 and zone 3 (goal zone). By contrast, most *cat-2* mutant worms lagged near the center of the plate, with peaks lingering in starting zone and zone 1, for at least 2 minutes before crawling efficiently toward the edge of the plate (Fig 1 *D*). Analysis of worm distribution at 120 seconds after emerging from the puddle (Fig 1*E*) revealed that a significantly greater fraction of wild-type worms

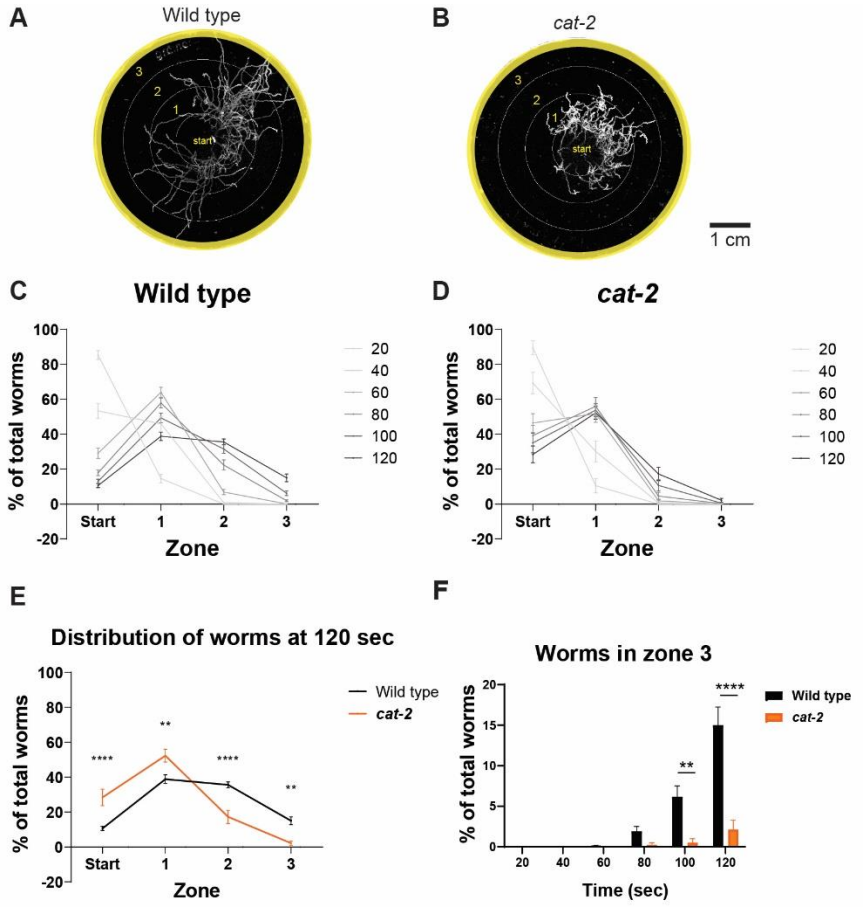

Fig 3.1. - Following swim-to-crawl transition, *cat-2* forward locomotion and displacement are reduced compared to wild type.

The radial dispersion assay is used to measure worm displacement from the start zone in the center of the plate. Worms are motivated to migrate toward zone 3 at the outer edge of the plate by the presence of a chemical odor attractant. (A) Representative example of the tracks made by a group of wild-type worms that participated in radial dispersion. Tracks were generated by creating a time-lapse image of frames recorded over a two-minute period after worms emerged from the central puddle. White circles are the borders between each zone. The yellow circle represents the placement of the attractant, diacetyl, which is spread evenly along the perimeter of the plate. (B) Representative example radial dispersion assay with the *cat-2* mutant strain. (C) and (D) The average percentage of worms in each zone is shown at each timepoint on separate lines. Radial dispersion trials using wild-type (N = 35) and *cat-2* worms (N = 15) consisted of 50-300 individual worms per trial. Worms in each zone were counted at 20 second intervals following the emergence of the first worms from the puddle. (E) Comparison of wild-type and *cat-2* worms in each zone at the 120 sec timepoint only. The distribution of wild type is shifted significantly toward zone 3 compared to *cat-2*, which largely remains in the inner zones. (2-way ANOVA, strain x zone, with post-hoc analysis using Bonferroni multiple comparisons test. Wild type *vs cat-2* in zone 3 at 120 sec, p = 0.0036). (F) Comparison of worms in zone 3 at all timepoints. Relative to *cat-2*, a significantly higher proportion of wild-type animals reached the outermost zone as early as the 100 sec timepoint (2-way ANOVA, strain x timepoint, with post-hoc analysis using Bonferroni multiple comparisons test. Wild type vs *cat-2* in zone 3 at 120 sec, p = 0.0019). This difference was further increased at the 120 sec timepoint (p<0.0001). All data points here and henceforth represent mean ± s.e.m unless otherwise specified.

(15.01% ± 13.2) reached the outer diameter of the plate than *cat-2* mutant worms (2.1% ±

4.4) after 2 minutes (Two-way ANOVA, strain x zone, with post-hoc analysis using Bonferroni multiple comparisons test , $p = 0.0036$). We also used the video to qualitatively compare how *cat-2* transitioned from swim to crawl versus wild type. We found that as *cat-2* worms emerged from the liquid they very briefly (~2-3 sec) moved at a normal speed, then suddenly came to a halt, as if hitting an invisible wall. Following this halt, individual *cat-2* mutants frequently displayed at least one of two other distinct behaviors as they attempted to initiate crawling; (1) an unusual posture during attempts to crawl forward, and (2) a noticeable increase in reversals. During attempts at forward crawling, dorsoventral bends initiated at the head failed to propagate the full length of the body. This appeared to impede forward progress as the posterior body remained flaccid. Interestingly, not only did the worms subsequently begin to initiate a bout of reversals, but the body posture and motion during reversal appeared normal; bends initiated from the tail successfully propagated along the full length of the body. These qualities suggest that the dopamine-dependent deficit is more specific to crawling forward than backward.

With these results we confirmed our previous finding that the *cat-2* mutant displays a delay in initiating crawling after swimming. In doing so we also established that our new radial dispersion assay is sufficient for evaluating swim-to-crawl transition efficiency and general locomotion patterns.

Mutations suppress the swim-to-crawl motor-transition deficit in a *cat-2* background

With our new population-based assay to help distinguish between efficient and inefficient swim-to-crawl motor transitions, we set out to perform a forward genetic screen

to isolate mutations that suppress the motor transition phenotype of *cat-2*. For our suppressor screen we selected the *cat-2* mutant strain carrying the allele *tm2261*. This knockout mutant harbors a 211 bp deletion in an obligate exon of the *cat-2* gene, replaced by a 3 bp insertion. By using a deletion this size we would be unlikely to recover intragenic revertant mutations from our suppressor screen, and be more likely to recover mutations in other genes that contribute to motor transitions. In this particular null mutant, dopamine was undetectable by the formaldehyde-induced fluorescence (FIF), a technique sometimes used in *C. elegans* for dopamine detection (National BioResource Project). Dopamine was detected at low levels (~10% of wild type) by high-performance liquid chromatography (HPLC) (Hardaway et al., 2012).

As shown in Fig 3.1, the first of the wild-type worms reach the outermost region of the plate (zone 3) just after the one-minute mark (2.0% ± 3.5). By the 100-sec (1.66 min) timepoint an average of 6.15% ± 8 of wild-type worms reach zone 3. By contrast, *cat-2* worms typically require two minutes or more to reach this zone, and still an average of only 2.14% ± 4.4 are in zone 3 at this timepoint. Therefore, we isolated individuals which reached zone 3 within 1.5 minutes or less. Selecting individuals at this timepoint minimized the likelihood of false positives for *cat-2* suppression. Each suppressor line that was generated from these individuals was later retested as a population in order to confirm that the line reliably reached zone 3 faster than *cat-2*. Of the 20 suppressor lines generated from this screen, we found only four that continued to perform reliably as expected (Fig 2). Henceforth, we refer to these strains by the unique allele names we assigned them (beginning with *vx*) in accordance with standard *C. elegans* naming conventions. The

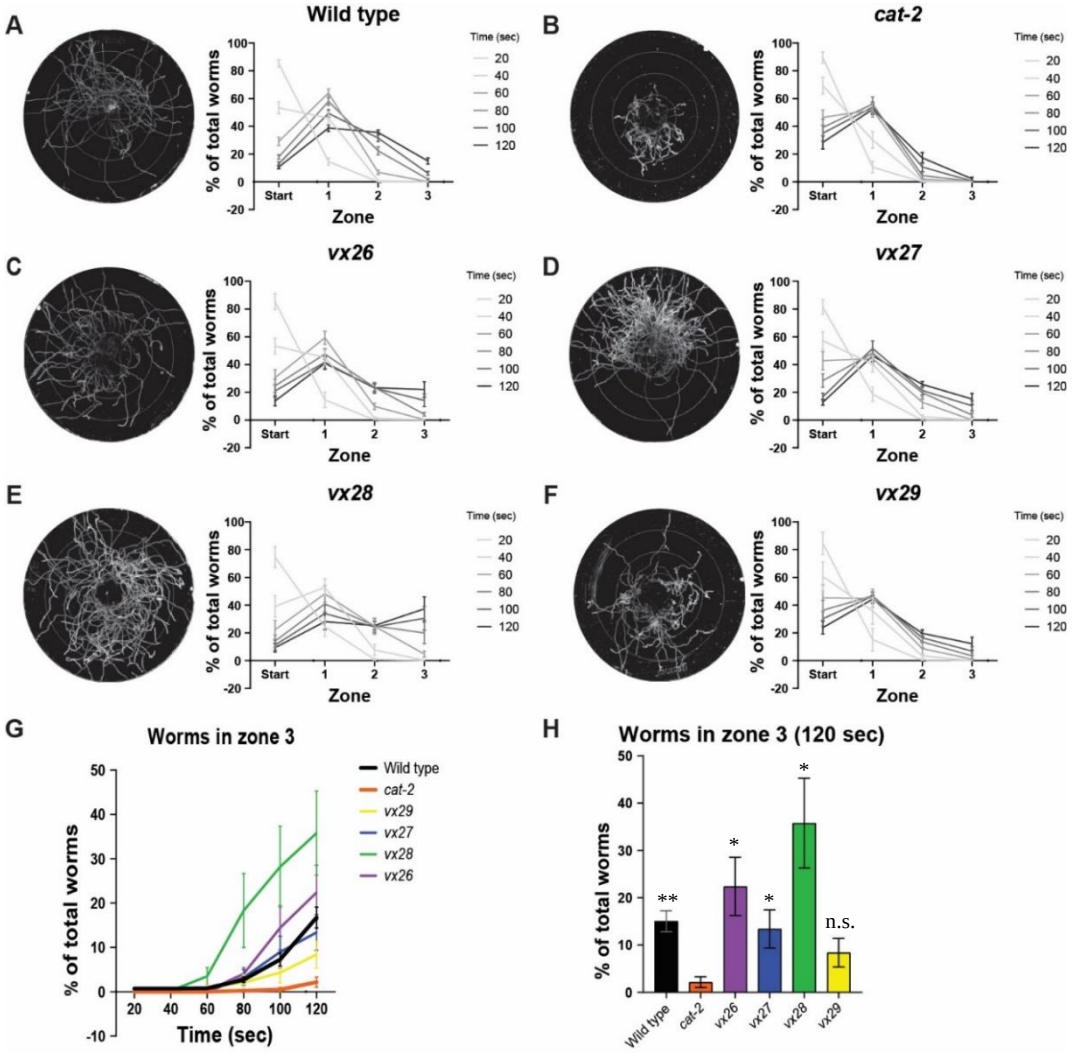

Fig 3.2 - Suppressor mutants perform as well as or better than wild type in radial dispersion assays. (*A* – *F*) Representative examples of tracks from radial dispersion trials for each strain (left). Graphs to the right of these images show the average percentages of worms in each zone at all timepoints for all strains, including wild type (*A*), *cat-2* mutants (*B*), and suppressor of *cat-2* mutants (*C* – *F*). (G) The average percentage of worms in zone 3 at all timepoints. These data were analyzed using two-way ANOVA with repeated measures, restricted to 80, 100 and 120 sec timepoints, where wild type and suppressor mutant strains were compared with *cat-2* (Dunnett method for post-hoc multiple comparisons test). Earlier timepoints were excluded because no *cat-2* worms were present in zone 3, therefore comparisons could not be made. At 80 sec, only average percentage of *vx26* in zone 3 was significantly higher than that of *cat-2* (N = 11, p = 0.047). At 100 sec, strains that differed significantly from *cat-2* were wild type (N = 35, p < 0.0028), *vx26* (N = 11, p = 0.039), *vx27* (N = 10, p = 0.043), and *vx28* (N = 10, p = 0.0179). (H) The average percentage of worms in zone 3 at only the 120 sec timepoint. At this timepoint, strains that differed significantly from *cat-2* were wild type (N = 35, p < 0.0001), *vx26* (N = 11, p = 0.0176), *vx27* (N = 10, p = 0.0247) and *vx28* (N = 10, p = 0.0099).

average percentage of worms that have reached Zone 3 is plotted in Fig 3.2 *G*, with those

in zone 3 at the final timepoint, 120 sec, in Fig 3.2 *H*. We found that, like wild type, each of the suppressor strains had the first individuals reaching zone 3 at the 80 sec timepoint.

By the final two time points three of the suppressor strains showed significantly higher numbers in zone 3 that *cat-2* (3.2 *G* and *H*). All but one of the suppressor strains differed significantly from *cat-2* in reaching zone 3 by the end of the radial dispersion race. Notably, *vx28* even often surpassed wild type in reaching the outer zones.

Suppression of *cat-2* deficits is not due to locomotor hyperactivity

One possible mechanism through which these mutants may be able to migrate at similar rates to wild-type worms is simple locomotor hyperactivity. To test this possibility, we repeated the radial dispersion assays without the swim-to-crawl transition. In other words, we performed a "dry" radial dispersion by picking worms to the center of the plate with a platinum spatula. Contrary to the locomotor hyperactivity explanation, the dry dispersion results revealed similar locomotor rates across all

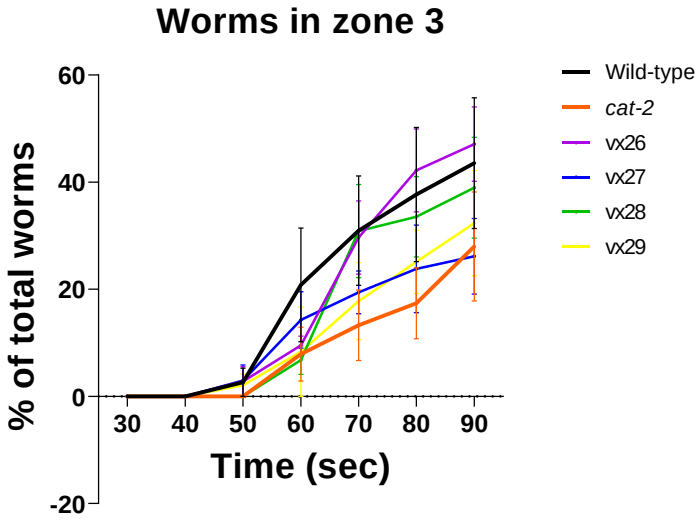

Fig 3.3 - Improved radial dispersion performance is not due to an overall increase in speed.
Radial dispersion assays repeated without requiring swim-to-crawl transition. Instead of emerging from a puddle, worms are transferred directly onto the center of the agar of the essay plate. Worms were recorded from 30 seconds post-transfer to 90 seconds post-transfer. No difference in the percentage of worms in zone 3 was observed between any of the strains tested at any timepoint (2-way ANOVA using Bonferroni multiple comparison test) . N = 4 for all strains except *vx29*, for which N = 3.

groups. All mutants including *cat-2* reached zone 3 on a timeline comparable to wild type (Fig 3.3; 2-way ANOVA using Bonferroni multiple comparisons test). This finding suggests that the improved radial dispersion of the suppressor mutants compared to the *cat-2* parent strain stems instead from a change in the swim-to-crawl transition, as this was likely responsible for the delay in migration of *cat-2* in this assay.

In order to further characterize the suppressor mutants, with particular interest in dopamine-related phenotypes, we employed two contrasting manipulations of dopaminergic signaling pathways. First, we assessed how the mutants behaved with dopamine restored by treating with exogenous dopamine. These mutants are able to transition from swim-to-crawl efficiently in the near-absence of dopamine. However, without sequencing, the mechanisms that allow them to do so is unknown. By evaluating radial dispersion behavior in these strains after exogenous dopamine treatment we may uncover clues about the underlying mechanism of *cat-2* phenotype. For instance, if the suppression is the result of a mutation that causes the D1-like dopamine receptor or downstream targets to be active in absence of dopamine, we would expect that suppressor mutant to respond to the exogenous dopamine in a similar manner to wild-type worms, which would is dopamine-induced paralysis. If the mutation leads to dopamine signaling that is even higher than that of wild type, the would expect the mutant to be hypersensitive to dopamine.

We also investigated how suppressor mutants behaved after destruction of dopamine neurons by treatment with neurotoxin 6-hydroxydopamine (6-OHDA). Because

the suppressor mutants should be unable to produce dopamine, our prediction was that treatment with 6-OHDA should not have an impact on swim-to-crawl transition.

Exogenous dopamine treatment had variable effects suppressor mutant lines

Because the suppressor mutants are apparently able to efficiently transition from swim-to-crawl in absence of dopamine, we wanted to investigate how they behaved when supplemented with dopamine. We did this by treating by soaking the worms in an exogenous dopamine solution prior to radial dispersion testing. We have previously found that treatment with 25mM exogenous dopamine is sufficient to significantly improve swim-to-crawl transition in *cat-2* mutants (unpublished). In wild-type worms, this concentration of exogenous dopamine and higher is known to induce paralysis (Chase, Pepper, & Koelle, 2004). Based on these previous findings, we expected similar responses in *cat-2* and wild-type worms when treated with exogenous dopamine.

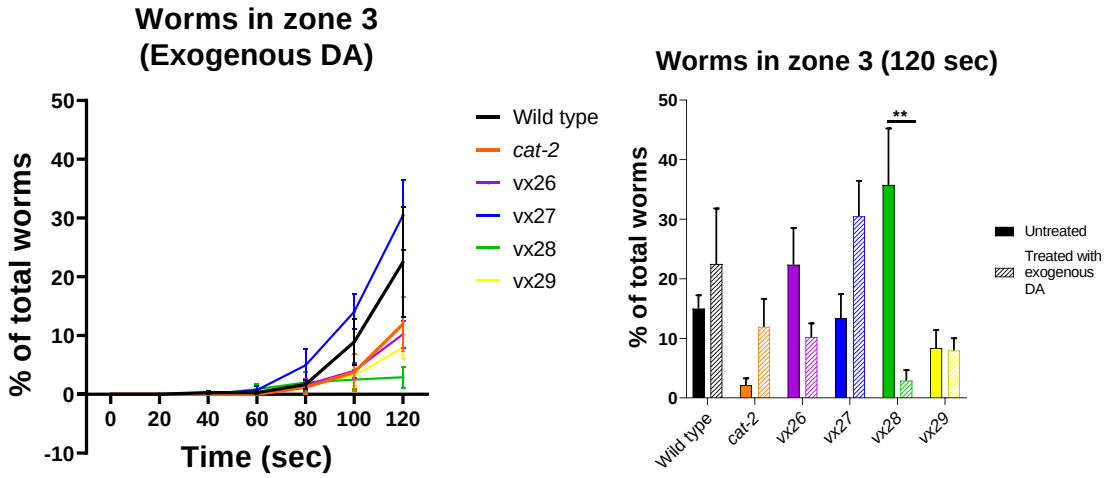

Fig 3.4 - Treatment with exogenous dopamine dramatically reduced locomotion in one suppressor mutant.
Radial dispersion assays following treatment with exogenous dopamine. (A) Average percentage of total exogenous DA-treated worms in zone 3 at each timepoint. (B) Average percentage of exogenous DA-treated worms and previously tested untreated worms in zone 3 at 120 sec.

As expected, the average percentage of *cat-2* worms that reached zone 3 after 120 seconds was higher after exogenous dopamine treatment than in the previous untreated trials (Fig 3.4 B). Though this result was not statistically significant (Welch's t, p = 0.105, N = 5), there is a clear trend toward an increase in *cat-2* reaching zone 3, which would likely be verified with additional trials. Contrary to our expectations, wild type did not appear to suffer from dopamine induced paralysis in these trials, and may have even reached zone 3 a bit faster than in untreated trials. However, this is likely due to the shorter duration of exogenous DA treatment compared to methods in Chase, et al 2004 (20 minutes in DA in their study vs ~10 minutes here). The most notable change in DA-treated versus untreated groups was observed in *vx28*. This strain had the highest average percentage of worms in zone 3 at 120 seconds in untreated radial dispersion trials, even exceeding wild type. The fact that exogenous dopamine produces a sizeable reduction in worms reaching zone 3 is likely the result of hypersensitivity to dopamine-induced paralysis. The mutant strain *vx26* showed a similar, but not quite significant, reduction, also suggesting a dopamine hypersensitive phenotype. In contrast these two mutants, a larger percentage of dopamine-treated *vx27* worms reached the outermost zone than in untreated trials. The direction of this trend indicates that the treatment enhances either the efficiency of the swim-to-crawl transition or locomotor speed in general. Finally, *vx29* showed no significant difference between the treated and untreated conditions.

6-OHDA did not greatly impair radial dispersion performance in suppressor mutants.

As expected, 6-OHDA treatment significantly impaired radial dispersion performance in wild type animals. Without a source of dopamine, wild type performance is comparable to that of *cat-2* and other dopamine deficient mutants previously studied (A. Vidal-Gadea et al., 2011). Results from *cat-2* after 6-OHDA treatment are omitted from figures because none of the *cat-2* worms reached zone 3 in the trials we completed. Comparison of treated versus untreated suppressor mutants did not reveal a significant change in radial dispersion, although this may be due to relatively small sample size (see Fig 3. 5). However, there was a strong trend observed in the results *vx26* suppressor mutant (p = 0.0526). This mutant appears to have performed better in the 6-OHDA treated condition than in the untreated condition. It was also the only strain that that showed a change in this direction after

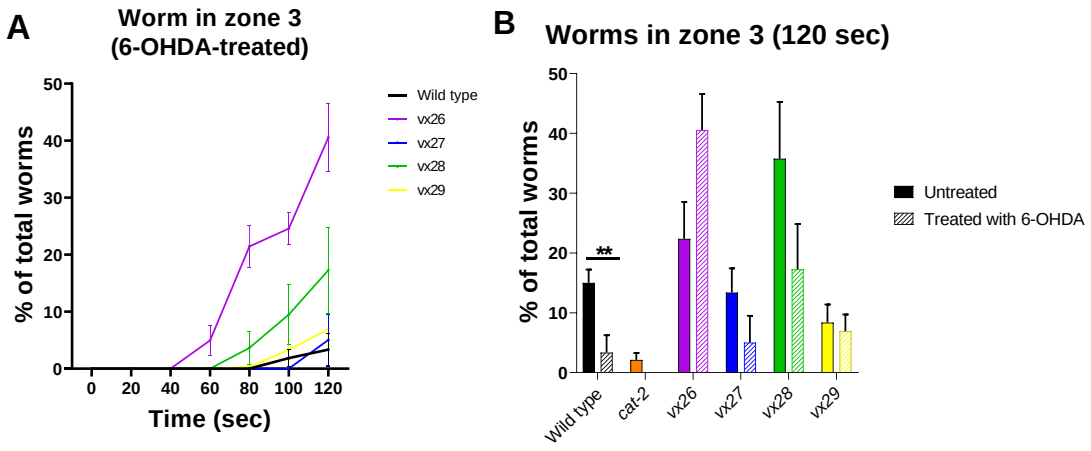

Fig 3.5 - Destruction of DA neurons by 6-OHDA had opposing effects in different suppressor mutants.
Radial dispersion assays using worms that have been previously treated with 6-OHDA. (A) Average percentage of total 6-OHDA treated worms in zone 3 at each timepoint. (B) Average percentage of exogenous 6-OHDA treated worms and previously tested untreated worms in zone 3 at 120 sec. 6-OHDA-treated vs untreated radial dispersion performance was evaluating using percentages in zone 3 at the 120 sec timepoint only, and each strain was compared to itself (Welch's t). Wild type (N = 6, p < 0.0079), *cat-2* untreated (N = 15), *vx26* (N = 4, p = 0.0526), *vx27* (N = 5, p = 0.1214), *vx28* (N = 5, p = 0.1062) and *vx29* (N = 5, p = 0.3511)

treatment with 6-OHDA. With a sample of only N = 4 additional trials will be necessary to confirm this result.

DISCUSSION

In this study we established a radial dispersion assay as a new and reliable way of quantifying the swim-to-crawl motor deficit exhibited by dopamine-deficient worms. We did so by reproducing the finding that the *cat-2* mutant experiences a brief period of immobility after emerging from a puddle of liquid. As a result, *cat-2* mutant worms are not able to cover as much distance when migrating away from the starting point as wild-type worms. This is now easily measured by the radial dispersion assay. With this new tool, we isolated individual worms from a forward genetic screen that overcame this phenotype of *cat-2*. From these individuals we generated four new *cat-2* strains that each harbor mutations that suppress the swim-to-crawl defect.

After generating these suppressor mutant strains, we next sought to uncover clues as to the mechanisms that underlie the suppression of this *cat-2* phenotype. We suspect there are several different potential suppression mechanisms. The *cat-2* mutant lacks the tyrosine hydroxylase enzyme, which is used to synthesize L-Dopa, a necessary precursor of dopamine. Without tyrosine hydroxylase, this mutant produces very little dopamine (Hardaway et al., 2012). Dopamine receptors, however, remain intact. Dopamine receptors in *C. elegans* fall into two main categories, D1-like and D2-like receptors, based on their functional similarity to mammalian dopamine receptors. Dopamine receptors in mammals and in C. elegans are G-protein coupled receptors, which have opposing downstream effects on the cell. Specifically, D1-like receptors (D1Rs) produce a generally excitatory effect while D2-like receptors (D2Rs) produce an inhibitory effect. Together, these

dopamine receptors work both synergistically and antagonistically toward each other to modulate locomotor activity in the worm (Chase et al., 2004). As previously described by our lab, D1R knockout mutants, but not D2R mutants, exhibit the same swim-to-crawl deficit as *cat-2* mutants (Vidal-Gadea, Pierce-Shimomura, 2012). Therefore, mutations affecting dopamine receptors and downstream components of their signaling pathways are reasonable candidates for suppressors of *cat-2*. For example, a mutation that leads to constitutive activity of the D1Rs, the associated G protein $G\alpha_{q,}$ or components downstream in this pathway might be sufficient to overcome the immobility *cat-2* during the swim-to-crawl transition. Mutations that could cause this could include 1) a mutation of the G-protein alpha subunit that allows it to more readily exchange GDP for GTP, making it prone to being activated in the absence of D1R ligand binding or 2) a mutation that decreases GTPase activity or otherwise prevent GTP hydrolysis, which deactivates the G-protein alpha subunit. This type of mutation would prolong the effects of the active subunit.

Another hypothetical D1R-related mechanism that may lead to suppression is a mutation of the receptor itself that alters D1R ligand specificity, making them receptive to activation by catecholamines, such as octopamine and tyramine. Octopamine and tyramine are biogenic amines that, like dopamine, are synthesized from tyrosine (Chase & Koelle, 2007). They are used as neurotransmitters in invertebrates and bear similarities to the neurotransmitters adrenaline and noradrenaline (Roeder, 2005). These alternative ligands could then restore D1R signaling activity to wild-type levels or even surpass them, also restoring effective transitioning. The function of these monoamines in worms is not yet fully understood, so this theory is speculative, based on structural similarity to dopamine.

Another factor to consider is that *cat-2* mutants retain some small amount of dopamine. Results from HPLC analysis revealed very low levels (<0.1ng DA/mg protein) of dopamine in the *cat-2* strain we used here (Hardaway et al., 2012). In another *cat-2* null mutant, *cat-2(e1112)*, dopamine is reduced to around 40% of that of wild-type animals (Sanyal et al, 2004). Since the *cat-2* null mutants lack the tyrosine hydroxylase (TH) enzyme needed to synthesize L-Dopa, and, in turn, dopamine, it has been hypothesized that L-Dopa may be synthesized in the worm instead by the tyrosinase enzyme (Sanyal et al., 2004; A. G. Vidal-Gadea & Pierce-Shimomura, 2012). This enzyme, involved in melanin synthesis in mice, was found to be the source of L-Dopa production in TH-null mutant mice (Rios et al., 1999). Tyrosinase synthesizes L-Dopa less efficiently than TH. There are 6 putative tyrosinase genes found in *C. elegans* ("Tyr (gene_class)—WormBase: Nematode Information Resource," n.d.), raising the possibility that a mutation in one of these genes could lead to increased L-Dopa synthesis. With this, dopamine production may be fully restored or even increased beyond wild-type levels, which would be consistent with the radial dispersion of some of the suppressor mutants.

Looking beyond molecular pathways directly involved dopamine signaling, mutations in other neural circuitry are also potential sources of swim-to-crawl defect suppression. For example, in conjunction with dopamine, gait initiation and maintenance are also modulated by serotonin. Previous, our lab established that these two monoamines had opposing effects on locomotory gait (Pierce-Shimomura et al., 2008; A. Vidal-Gadea et al., 2011). Specifically, serotonin, acts to initiate swimming in the worm, in contrast to dopamine, which acts to initiate crawling. Efficient transition between these gaits appears

to require a particular ratio of these two neurotransmitters. For example, in water, serotonin is increased while dopamine is inhibited. On land, the relationship is vice-versa. In water, bouts of slower, crawl-like movement were induced either by adding exogenous dopamine or by reducing serotonin through genetic mutation. This demonstrates that disrupting the ratio of these two neurotransmitters during swimming leads to impairment of swimming gait maintenance. Conversely, dopamine deficiency hastens onset of swimming when worms enter a puddle. Furthermore, increasing serotonin exogenously caused a delay in crawl onset after emerging from water, not unlike what we see in dopamine-deficient mutants (A. G. Vidal-Gadea & Pierce-Shimomura, 2012; A. Vidal-Gadea et al., 2011). It is a reasonable hypothesis, then, that if serotonin interferes with initiation of crawling, then if it is decreased enough or eliminated completely, it could hasten onset to crawl even in the *cat-2* mutant, given the low levels of dopamine that remain, much like decreased dopamine hastens onset of swim. It would be easy to test this idea by testing for the presence of serotonin in dopamine mutants using immunostaining techniques (Loer and Kenyon, 1993) or HPLC.

In an attempt to uncover clues as to how swim-to-crawl efficiency was recovered in these mutants, we treated worms with exogenous dopamine. The mechanisms of suppression that would be most easily identifiable using this method are those that increase dopaminergic signaling, such as a constitutively active or overactive D1R pathway, as in the first set of mechanisms we propose above. If this class of mutation is at work, the mutant would likely be hypersensitive paralysis by exogenous dopamine. This would also occur if the tyrosinase hyper-efficiency mechanism was at work in the mutant. On the other

hand, if radial dispersion performance is enhanced following exogenous dopamine treatment, a mutation outside of the dopamine pathway, such as one that decreases serotonin as we described, is more likely. In this scenario, the serotonin reduction would have been sufficient to restore transition, and additional dopamine would further enhance the dopamine-to-serotonin ratio needed to trigger crawl initiation.

The exogenous dopamine treatment results suggest that two of our four suppressor mutants may exhibit hypersensitivity to dopamine. The concentration of dopamine we used in this treatment (25 mM exogenous) has been previously shown to induce paralysis in wild-type worms (Chase et al., 2004). However, we did not see paralysis in the wild-type animals we tested. This is likely due to the shorter duration of dopamine treatment we used. We have also previously found that this concentration of exogenous dopamine improves swim-to-crawl transition in *cat-2* mutants significantly (not shown here). Two of the suppressor mutants, *vx26* and *vx28*, showed reduced performance in radial dispersion after dopamine treatment (though only the effect on *vx28* was significant). Given that wild-type animals did not display paralysis with our treatment protocol, these data strongly suggest that *vx26* and particularly *vx28* are hypersensitive to the effects of dopamine. This may be the result of one of the mechanisms that increase dopamine signaling we describe above, including tyrosinase hyper-efficiency and D1R overactivity. Given that *vx28* had the highest percentage of worms in zone 3 at the end of the radial dispersion assay, these overactive dopamine pathway mechanisms seem very plausible. Additional input from exogenous dopamine, therefore, could lead to hypersensitivity to dopamine paralysis. One mutant, *vx27*, showed a change in radial dispersion performance in the opposite direction,

actually increasing the percentage of worms that reached zone 3. This mutant may fit the reduced serotonin hypothesis described above. Another possible explanation for improved performance with exogenous dopamine is that *vx27* carries one of the mutations previously found to be resistant to dopamine-induced paralysis. Mutations that either decrease *dop-3* (a D2R) downstream signaling or increase *dop-1* (a D1R) downstream signaling showed resistance to dopamine-induced paralysis (Chase et al., 2004). In the *cat-2* background there may be enough dopamine to cause such an imbalance between D1R and D2R signaling if one of these mutations, making them good suppressor mutation candidates as well.

In our last set of experiments, we tested the hypothesis that suppressor mutants should maintain normal radial dispersion performance after dopamine neurons are destroyed. Since they are able to transition to crawling even with severely impaired dopamine production, the absence of the dopamine neurons should be inconsequential. Impaired performance of wild-type worms treated with the neurotoxin 6-OHDA confirmed that the concentration we were using was effective. Among the suppressor mutants there was no significant difference between the treated and untreated trials. Although this is consistent with our hypothesis it should be noted that *vx26* showed a strong trend toward improved radial dispersion performance. More trials of 6-OHDA-treated suppressor mutants are needed to clarify these results.

Ultimately, we would like to determine the specific mutations in the *ctsp* mutants using whole-genome sequencing to determine which molecular pathways may be tweaked to alleviate dopamine-deficient motor defects. Once we have determined which mutations

specifically are responsible for *cat-2* suppression in these mutants, we can test other mutant alleles of the same gene, this time without the many background mutations carried by our suppressor lines. If the mutation is in a previously undescribed gene, we will also have the opportunity to close and characterize it for the first time. This process requires dominance testing, complementation testing, and several rounds of backcrossing before submitting strains for sequencing. In the meantime, further characterization of these suppressor mutants may help us to pinpoint the genes or class of genes responsible for *cat-2* suppression.

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CHAPTER FOUR: FINAL THOUGHTS AND FUTURE DIRECTIONS

PART 1

In this study I set out to investigate the effects of ancestral chronic EtOH exposure on subsequent, EtOH-naïve generations. With mounting evidence for transgenerational epigenetic inheritance (TEI), and especially promising recent evidence in worms, I was eager to pursue this study because there were several aspects of this project that had not been attempted by either myself or other lab members. Working through the trial and error that comes along with developing a new behavioral assay can be frustrating, but I also found it rewarding. That said, now that I am wrapping up and reviewing this work, I see that there are still some residual kinks that need to be ironed out before this work is picked up again.

Addressing likely confounds

If I were able to continue with this research, my next goal would be to investigate the only truly significant effect I found during the transgenerational study: the effect of season on the speed of the worms being tested. Season not only had a significant effect on the baseline speed of the worms, it also had a significant effect on their intoxicated speed, which was the measure of interest in this set of experiments. More importantly, season did not have a uniform effect on both conditions. For example, when comparing spring speeds to summer speeds, the discrepancy between the two seasons is larger for the baseline condition than the On-EtOH condition. In order to be confident in future results from

transgenerational work such as this, this confound will need to be addressed. This is not only a concern for transgenerational work, but probably for any behavioral work carried out in laboratories that are so susceptible to seasonal condition confounds. The best way to go about investigating a solution to this would be to begin keeping daily records of certain useful measures. Temperature and humidity are obviously the two top priorities to measure daily, but I would not limit the scope of the "investigation" to only these. The agar assay plates used in the lab can be notoriously sensitive to changes in ambient conditions. Besides measuring lab conditions, testing possible solutions to minimize sources of variability is also necessary, such as using an incubator or other climate-controlled space for raising and testing worms.

Another potential confound I would want to address is how starvation contributed to the EtOH-line phenotypes. The chronic EtOH-treatment paradigm requires that worms be placed on a treatment plate with EtOH for 24 hours, continuously. The control line, which is run in parallel, spend the same 24 hours under typical lab condition, on a "mock treatment" plate, which has only had a small amount of water added under the agar to mimic the EtOH plate handling. A major side effect of the EtOH treatment is that it reduces the worm's pharyngeal pumping rate. This is the rate at which the worm is takes in food from the surrounding bacterial lawn. Besides the physical impediment preventing the worm from eating, the odor or flavor of the EtOH may be too aversive, causing it to avoid feeding. We can say with certainty that the worms left on EtOH for 24 hours do experience a period of severe caloric restriction. This is evidenced by the noticeably reduced side of the EtOH-line worms at the end of the 24-hour treatment. Findings from other recent *C. elegans*

research suggests a non-trivial effect of starvation on offspring. Worms put on dietary restriction during adulthood had fewer early progeny (small brood size) but embryos were larger and L1 larvae were also larger. L1 larvae were also starvation resistant (Hibshman et al., 2016). We had already been somewhat concerned about the possibility of the EtOH treatment itself resulting in reduced fecundity, as it could result in unplanned selection of F1 embryos. Although we did not quantify fecundity during this study, I did suspect that early brood size of the EtOH-line P0 generation might be reduced, just based on casual observation. I did not note a different in embryo or larva size of the progeny. With this information, I would be curious to know if the small brood size I thought I saw in the EtOH-line was in fact due to the starvation effect, but not the EtOH. I think it would be worthwhile to repeat the P0 and F1 leg of the transgenerational study, this time with two control-lines and one EtOH-line in the P0 generation: One well fed control-line (no different than our original control-line), and one starved control-line, which will be placed on an unseeded "mock treatment" plate for the duration of the 24-hour treatment period, followed by brood size measurement for 2 to 3 days. Carrying out the acute intoxication assays again in the F1 generation, this time alongside a starved control, may indicate if any starvation related phenotypes contributed to EtOH-line behavior.

Lessons from RNAi silencing

After collecting the earliest data from this project, which, at that time, only included the chronic EtOH treatment, I was excited to see that the EtOH line showed a slight

resistance to acute intoxication. After that it almost seemed as though every other cohort I ran would pull my EtOH-line effect back down below significance, only have the next one give me hope again. Reading more into TEI studies in *C. elegans*, I began to suspect that epigenetic inheritance, as in, inheritance of an epi-allele, has an inherent stochasticity to it. That is, an environmental trigger could (and probably does) act on the parental generation by producing some degree of epigenetic response. This parental response may or may not produce some phenotypic readout in the offspring, and, importantly, individual offspring may be affected differently by parental exposure. If the parental exposure does result in some phenotype in an F1 individual, it may or may not be transmitted beyond this generation (intergenerational inheritance). Yet, in some (probably less frequent) cases, the environmental trigger can lead to transmission of a phenotype beyond the F1 generation. From here, stable transmission of what could be considered an epi-allele is dependent on some form of maintenance of the conditions producing the epi-allele, like chromatin state, for example. This stochasticity is illustrated nicely in the schematic model for transgenerational epigenetic memory laid out in the 2014 review by William Kelly.

This idea is consistent with some of the features of RNAi inheritance in *C. elegans*. For example, although RNAi silencing can be transmitted to progeny via TEI, there are some caveats. First, RNAi has variable penetrance (Vastenhouw et al., 2006). Since RNAi mechanisms of heritable gene silencing overlap greatly with that of endogenous siRNA (endo-siRNA) in the worm, it is possible that endo-siRNA can induce TEI in response to environmental perturbations with similarly variable penetrance. This could explain why

we saw little to no effect of EtOH-treatment in the F1 generation, as low penetrance of an EtOH-induced phenotype may have caused it to be overlooked.

The most long-term transgenerational transmission (>80 generations) of RNAi silencing demonstrated in the Vastenhouw study was only achieved by repetitive selection of individuals that showed silencing of the target gene (in this case, a GFP transgene). Given the variable penetrance of silencing, it makes sense that selection is required in order to extend and maintain transgenerational silencing, otherwise the silenced phenotype would eventually be lost. If I am correct in my assumption that parental EtOH-induced heritable phenotypes are also variably penetrant, selection may be necessary to see the effect of EtOH resistance we expect and have seen in some cohorts. In the hypothetical scenario that parental EtOH-treatment gives rise to an EtOH-resistant phenotype in 30% of progeny (I use this figure because it was the level of penetrance describe in a representative example of RNAi silencing), testing a random sample of the EtOH-line progeny would likely result in "wash-out" of the phenotype. I think it is reasonable to suspect a similar scenario in our past experience. To test this, a selection protocol would need to be carried out in the F1 generation. To avoid bias of selecting and testing only resistant individuals, it would be best to instead divide F1 individuals into 3 categories based on resistance level, either resistant, sensitive, or neither. Criteria for selection into each category can be determined experimentally by analyzing the typical ranges of EtOH sensitivity across a large number of individual worms. It will also be necessary to harvest eggs from individual worms before testing for resistance, because, at least for this set of experiments, we are

only interested in testing worms with only a single ancestral EtOH trigger. However, there is also precedence for testing triggers in consecutive generations.

Resistance selection is one method that may allow future transgenerational studies such as this one to uncover variably penetrant transgenerational phenotypes. However, there may be other unexplored methods of delivering environmental triggers that enhance TEI in the worm. RNAi-induced TEI may provide more clues to promising approaches and treatment paradigms. For example. multiple ancestral triggers were also found to both extend duration and enhance potency of transgenerational RNAi silencing (Houri-Ze'evi et al., 2016). That is, in an RNAi silencing model targeting GFP, GFP fluorescence gradually returned to baseline levels within 4-5 generations. If and RNAi trigger was administered in the F1 generation, however, the GFP fluorescence knock-down persisted for longer and to a greater degree. Intriguingly, the second trigger used was not the same dsRNA used in the first trigger (against GFP), but succeeded in enhancing the transgenerational silencing of GFP. This suggests that repeated exposures are an important area to explore in transgenerational inheritance research, especially since real-world environmental exposures often persist across more than one generation. This is a concept we have been interested in testing for some time, and these RNAi silencing findings provide further justification for investigating consecutive epigenetic triggers.

There are of course a multitude of other directions to explore related to this initial study. It is not clear yet what specific conditions must be in place for TEI to occur following specific environmental perturbations, or if some triggers are capable of producing a

transgenerational effect at all. I would personally prioritize the resistance selection experiment and the consecutive trigger experiment. Other approaches I have considered exploring include male parental EtOH exposure, given some of the advantages discussed in the introduction section, and testing various EtOH-treatment schedules that compare the variables of developmental timing, duration and frequency of EtOH exposure. We have some initial results from the latter, using and intermittent EtOH treatment, but this should be repeated and also compared with other treatment schedules.

As a final note, I realize that there is a limit to how well TEI mechanisms employed by *C. elegans* will translate to humans and mammals. However, there is a tremendous opportunity for expanding our understanding of an only recently discovered family of TEI mechanisms that are likely applicable to Drosophila and plant systems, as well as other species that may also use small ncRNAs and histone remodeling for transgenerational transmission. Furthermore, it may encourage an increased interest in investigating epigenetic mechanisms beyond DNA methylation in mammalian systems.

PART 2

The work I describe here in Chapter 3 of this thesis is a continuation previous work our lab members have carried out in order to elucidate the remarkably conserved role of dopamine in modulating locomotory gaits. One of our goals in doing so was to attempt to bridge the gaps in knowledge regarding gait modulation in invertebrates. Additionally, because of the extent to which some of the mechanisms we study here are conserved, it

also allows us to apply our findings more broadly to other animals and possibly even to human clinical research.

Here we exploited the large brood size and self-fertilizing hermaphrodites to carry out a forward genetic screen. With the way these screens are carried out in worms, using the chemical mutagen ENU, it is theoretically possible to induce mutations in each of the roughly 20,000 genes in *C. elegans*. Our F2 screen tested 50 plates containing several hundred worms each. Unlike some other screens, in which individuals are evaluated for the (hopefully easily visible) phenotype, this screen required testing worms as a population. Individuals that finished our radial dispersion "race" within the allotted 1.5-minute window where isolated and propagated.

While, in some ways, population testing made the screen easier, the fact that we were screening for a phenotype that is definitely not black-and-white left some room for a few false positive "hits," and many more false negatives. That is, although *cat-2* mutants very rarely reach zone 3, it can certainly happen, especially when screening upward of 20,000 individuals. The possibility of false positives, however, was combatted by retesting potential suppressor strains in several generations to confirm consistent performance. It is likely, however, that this screen missed individual suppressor mutants due to the fact that even in wild-type worms only about 5% of individuals finish the race by 1.5 minutes. Any given wild-type worm is capable of efficient swim-to-crawl transition, ensuring separation between wild-type and *cat-2* worms required use of a specific, narrow window for selection. So, while the mutagenesis itself produced a semi-exhaustive assortment of possible mutants, the nature of the phenotype and population testing it requires imposes

some limitations on yield of this screen. Still, we were able to generate four mutant lines that seem to reliably suppress the inefficient swim-to-crawl phenotype, effectively "curing" a parkinsonian defect in *C. elegans*. An argument could be made, however, for carrying out another screen in the future in an attempt to uncover additional mutants that were missed in the first screen.

Suppressor mutants and their secondary phenotypes

We suspect that these four mutants each possess a unique suppressor mutation. This is, in part, because each mutant exhibits some unique traits. It is important to note, though, that the secondary characteristics may either be attributed to background mutations or to the suppressor mutation of interest. Nonetheless, I found the secondary phenotypes of these mutants interesting and worth discussing here. These phenotypes may even contribute to the enhanced radial dispersion performance, regardless of whether they are caused by the suppressor mutation or a background mutation. The following are the more salient phenotypes we have noticed in these mutants.

The *vx26* mutant has a penchant for burrowing. For context, worms in the lab are raised on plates containing a flat layer of NGM agar. At room temperature, this gelatin-like material is rather firm with a solid surface that worms generally cannot penetrate. However, if the surface is punctured or damaged in some way, worms will quickly attempt to burrow inside, as burrowing is how they navigate their natural environment, soil, 3-dimensionally. The agar is typically too firm for the worms to burrow through, even if they gain a point of entry. These worms however, seem to burrow more efficiently and do so en masse, until

the agar becomes soft and malleable. It may be that these worms have enhanced muscle-tone, compared to most other strains. If so, it could play an important role in their improved radial dispersion performance as well.

Another interesting secondary phenotype we observed was in the *vx27* mutant. The animals invariably, regardless of age, congregate at the border of the bacterial lawn in mounds or clumps. These two behaviors have been studies in worms for some time now, and are attributed to both social feeding behavior (mediated by a gene called npr-1) and oxygen avoidance (mediated by genes in the oxygen-sensing pathway) (de Bono & Bargmann, 1998; Gray et al., 2004). In fact, the social feeding behavior may actually also be a form of oxygen avoidance, as the clumping and bordering associated with it can be induced by hyperoxic conditions. Incidentally, *cat-2* mutants tend to display mild bordering behavior as well, although this may be caused by the *cat-2* basal slowing defect. While wild-type worms reduce locomotor speed after entering the bacterial lawn (basal slowing), *cat-2* generally does not, or does so to a lesser degree. The border of the bacterial lawn, however, is thicker than the rest of the lawn. Since the reduced basal slowing in *cat-2* is likely due to a mechanosensory defect, preventing the animal from sensing the presence of the law, it is possible that the thicker border provides sufficient mechanosensory stimulus to slow *cat-2* worms (Nagarajan et al., 2014). Given the *cat-2* background of *vx27*, mild bordering would not be unexpected. However, this mutant displays a much more extreme form of clumping. They gather in large mounds, spaced remarkably equidistant from one another along the border. Only a small percentage of individuals are found elsewhere on the plate. It is not clear if this very salient behavior contributes to the radial dispersion

behavior of *vx27*, but I would be very interested in exploring this secondary phenotype further. Measuring *npr-1* and oxygen sensing pathway gene expression in the strain may shed some light on the cause of this behavior.

The *vx28* mutant stood apart from all other strain in the radial dispersion assay, reaching the zone 3 goal significantly faster than not only *cat-2* but significantly faster than wild type as well. The only noteworthy secondary phenotype we observed was reduced body bend amplitude in some individuals. This may not be quite as exciting as the secondary phenotypes described above, the main phenotype of *vx28* is certainly impressive.

Lastly, the *vx29* mutant showed the most modest rescue of the swim-to-crawl transition. While it did generally show higher numbers in zone 3 at the end of the radial dispersion races, this measure was not significantly different from that of *cat-2*. Furthermore, this mutant strain propagates slowly and individuals tend to grow and reproduce at different rates. It also seems to show reduced fertility which may decline across generations. We freeze each new worm strain we generate in order to preserve the original genetic background and avoid generational drift. This allows us to periodically thaw subsets of the strain as it was when it was created. If this mutant is thawed and propagated for several months, we see a decline in egg laying. In one instance the this even resulted in eventual sterility. Due to the modest radial dispersion performance and difficulty to maintain, I have considered leaving this mutant out of future experiments. However, before totally abandoning this strain it may worth attempting to backcross to *cat-2*. If background mutations are responsible for some of the problems we see in this mutant,

backcrossing may remedy the issues. If this mutant does indeed harbor a unique suppressor mutation it would be a waste if we prematurely rejected it.

Whole-genome sequencing and beyond

In working with the *cat-2* suppressor mutants, which we like to refer to as *ctsp* mutants (*cat-2* suppressor, aka catsup, as these mutants can "catch up" to wild type), our eventual goal is to prepare them for whole-genome sequencing (WGS). Several important steps are required. In a simple world, dominance testing will reveal whether the suppressor mutations are dominant or recessive, although realistically a more complicated relationship may be involved, such as incomplete dominance. If we find that our mutations are recessive, we can carry out complementation testing, which will inform us if any of the suppressor mutants harbor a mutation in the same gene. If any are in in the same gene it could save us the trouble (and cost) of sending both for sequencing. Finally, backcrossing is necessary for the WGS approach that incorporates SNP-mapping, because excessive background mutations will make analysis next to impossible (Doitsidou, Poole, Sarin, Bigelow, & Hobert, 2010). Each of these steps requires crossing, which is a complicated and very tedious processing with this set of mutants. If our phenotype was black-and-white and could be distinguished in each individual, selecting the correct offspring (i.e. the ones that have the suppressor mutation) after a cross would be fairly straightforward. But rescue of swim-to-crawl transition is a population-based phenotype that often requires repeated testing to confirm. It can be done, but it will require time and patience.

In the meantime, while we are carrying out preparations for WGS, there are several other useful questions to investigate using the suppressor mutants. For example, moving forward I think it would be worthwhile to observe and characterize the unique kinematics of each of the mutants during at they emerge from the receding puddle. Early in the Chapter 3 results section I describe, in detail, the kinematics of the *cat-2* mutant as emerges and attempts to initiate crawling, simultaneously comparing to the same behavior in wild type. Unique aspects of the *cat-2* swim-crawl transition include 1) incomplete propagation of the body bend wave to the posterior of the animal resulting in a partially flaccid posture, 2) dramatic increase in spontaneous reversals. Importantly, these reversals are carried out normally, with complete posterior-to-anterior body bend wave propagation, indicating that the posterior end is indeed capable of locomotion, but fails to do so in the forward directions. Because we used radial dispersion as a proxy for measuring swim-to-crawl transition, we do not actually know which, if any, of these kinematic features were restored to normal in the suppressor mutants. Our videos of radial dispersion lack the resolution necessary for evaluating worm body posture, we will need to observe each of the mutants as the immerge from liquid at a higher magnification. Kinematic analysis can be done either by manually scoring videos or by employing kinematic analysis software previously used by our lab members (Pierce-Shimomura et al., 2008; A. G. Vidal-Gadea & Pierce-Shimomura, 2012; A. Vidal-Gadea et al., 2011). This can be done relatively quickly and easily to answer the critical question of how the mutants are able to match (or surpass) wild-type worms in radial dispersion assays.

Our initial attempts to characterize suppressor mutants provided some clues as to the mechanism of suppression in some mutants. From here we can plan experiments to test our specific hypotheses, prioritizing those backed by experimental evidence. For example, exogenous dopamine experiments suggest that two of our mutants, *vx26* and *vx28,* may be hypersensitive to dopamine induced paralysis. We hypothesized that this could be causes by dopamine receptor signaling imbalances, or maybe even overproduction of dopamine through tyrosinases. These are easily testable. Pharmacological manipulation, using receptor specific antagonists can be used to determine whether or not the exogenous DA hypersensitivity is the result of D1R signaling, for example. RNAi knockdown is another potentially useful tool to investigate whether or not the D1R signaling is responsible for swim-to-crawl defect suppression in either of these mutants. Likewise, we can utilize FIF staining to detect the presence of normal levels of dopamine. If so, it may have been restored by tyrosinase activity, although further testing, such as detection using HPLC, may be needed to confirm this.

We also proposed a potential mechanism of suppression that is not directly involved in dopamine signaling – serotonin reduction. As described throughout this dissertation, there is substantial evidence the correct balance of dopamine and serotonin is necessary to execute and maintain swimming and crawling gaits in *C. elegans*. Crawl initiation specifically requires an increase in dopamine and inhibition of serotonin. Because dopamine is already reduced in the *cat-2* background, impairing crawl initiation after swimming, we hypothesize that an even more dramatic reduction of serotonin, and perhaps complete elimination of it, would restore the balance closely enough to allow crawl

initiation by mediated by dopamine. Importantly, we have the ability to test this idea without needing to use the suppressor mutants at all, by knocking down serotonin in the *cat-2* mutant. As with the dopamine receptors, there are several possible approaches to this, including pharmacological manipulation, genetic manipulation (crossing *cat-2* with a serotonin null mutant, for example), and knockdown with RNAi. Whichever manipulation we settle on using, radial dispersion will reveal whether or not our hypothesis that serotonin reduction can restore swim-to-crawl transition is plausible. As for the suppressor mutants themselves, immunostaining is a quick and simple way we can measure serotonin levels.

I think that testing each of these three leading hypotheses on mechanisms is a great next step in this project, and should at least be able to narrow down the possibilities by ruling some out. The results may even confirm one or more of these hypotheses, leading us the right direction to a suppressor mutation without the need for WGS. In either case, this project is rich with unexplored directions.

Finally, I sincerely hope that the work I have detailed in this thesis provides some meaningful contributions to further progress in both of these fields.

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